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**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**Inventors: Ecker, Griffey, Sampath, Hofstadler, and McNeil**

**Serial No.: 10/156,608**

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**Filed: May 24, 2002**

**Examiner: J. Fredman**

**Title: Method For Rapid Detection And Identification Of Bioagents**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Sir:

**DECLARATION UNDER 37 CFR §1.132**

I, Steven Buchsbaum, Ph.D., do hereby declare as follows:

1. I received an M.S. and Ph.D. in physics in 1990, and an M.P.I.A. with a specialization in International Technology Management in 1997, from the University of California, San Diego. I was a founding member and Director of the Office of Chemical, Biological, Radiological, Nuclear and Explosive Defense for the Department of Homeland Security's Advanced Research Projects Agency (HSARPA), created in 2003. Prior to joining HSARPA, I was a Program Manager in the Special Projects Office of the Defense Advanced Research Projects Agency (DARPA) where I was responsible for the development of: biosensors and defense systems against biological weapons; technologies to counter use of underground facilities; and other classified work.

2. TIGER (Triangulation Identification for the Genetic Evaluation of Risk) is an embodiment of a method of amplification of nucleic acid of a bioagent with a pair of primers that hybridize to nucleic acid of a wide range of bioagents at sequences which flank a variable sequence. Measurement of the molecular mass or base composition of the amplification product provides means to rapidly identify the bioagent without any prior knowledge or assumptions of the identity of the bioagent and without sequencing of the amplification product. I understand

that this methodology is disclosed and claimed in the patent application in connection with which this declaration is being submitted. TIGER represents a completely novel approach which is not obvious in view of previously existing technologies. To my knowledge, no one ever previously proposed or disclosed that combining broad range priming of nucleic acid of bioagents with molecular mass measurements would be successful in rapid and accurate identification of bacterial and viral bioagents. Thus, the invention of the TIGER method produces results that would be unexpected by simply combining existing technologies such as general broad range priming and mass spectrometry.

3. I was the original DARPA Program Manager for DARPA contract MDA972-00-C-0053 through which funds were granted via a subcontract to Ibis Therapeutics, a division of Isis Pharmaceuticals, for the provision of biosensors for broad-based identification of biowarfare agents and emerging infectious diseases in environmental and human clinical samples. As a DARPA program manager, it was my responsibility to invest in "high risk, high reward" concepts with a tolerance for failure. In the particular case of the Isis work, at the start of the project I felt that there was very high risk that their novel concept of broad-range identification of bioagents by molecular mass analysis, then in its infancy stage, would meet with success. In fact, an early internal review (funded by the DARPA director) carried out by JASON, an elite independent senior scientific advisory group that provides consulting services to the U.S. government on matters of defense science and technology, concluded that it was unlikely that development of the proposed methods would be successful. The unexpected success of the methods developed under the project was such that I nominated Ibis Therapeutics for an award for best performance under a DARPA contract.

4. I have been pleased to observe that over the last five years, Ibis Therapeutics has been awarded substantial additional funding from other U.S. government agencies including the Department of Defense, The Center for Disease Control, the National Institutes of Health, and the Homeland Security Advanced Research Projects Agency (HSARPA). Some of this funding was applied for at my suggestion and with my assistance while I was a DARPA Program Manager. While this grant funding has focused upon the long felt but unmet needs in biodefense,

infectious disease surveillance, and forensics, TIGER also has great applicability for broad application in other areas of healthcare, including pharmaceutical process control, healthcare-associated infection control, and *in vitro* diagnostics.

5. I have also been pleased to observe that several independent commentaries and high visibility publications in prestigious journals have provided an indication that the TIGER methods are innovative, produce results that would be unexpected in light of prior technologies, satisfy a long-felt and unmet need, and have great potential for commercial success. The appended exhibits (A-E) illustrate these points.

6. Exhibit A describes an embodiment of the TIGER method wherein a particularly virulent strain of *Streptococcus pyogenes* was identified as the cause of an outbreak of respiratory disease in a military barracks. This article appeared in the May 21, 2005 issue of *Proceedings of the National Academy of Sciences*. The article, independently edited and peer reviewed, indicates the innovative nature of the method and that the method satisfies a long felt need for rapid identification of infectious bioagents for epidemic tracking such as specific subtypes of *Streptococcus pyogenes* and other co-infecting respiratory pathogens. One particularly surprising and unexpected result of great utility that was obtained from the invention is that it may also be used for an analysis of microbial populations. For example, as described on page 8015, column 2 of the article, it is stated that "...military recruits in the midst of a respiratory disease outbreak had a dramatically different microbial population than that experienced by the general population in the absence of epidemic disease."

7. Exhibit B is a third-party article that appeared in the August 1, 2004 issue of *Analytical Chemistry* which describes the innovations of the TIGER method.

8. Exhibit C is a third-party "innovations article" which appeared in *Scientific American* in November 2002.

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9. **Exhibit D** is a third-party review article published in *Nature Reviews – Drug Discovery* in April 2005 which highlights the applicability of the TIGER method in the field of biodefense (see page 292, col. 2).

10. **Exhibit E** is a third-party article published in the August 27, 2004 issue of *Science* which indicates the applicability of the TIGER method as a biosensor for air surveillance of pathogens (see page 1229, col 1).

11. I declare that all statements made herein are of my own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

6/15/2005

Date

S. K. Budsh

Name

## EXHIBIT A



# Rapid identification and strain-typing of respiratory pathogens for epidemic surveillance

David J. Ecker, Rangarajan Sampath, Lawrence B. Blyn, Mark W. Eshoo, Cristina Ivy, Joseph A. Ecker, Brian Libby, Vivek Samant, Kristin A. Sannes-Lowery, Rachael E. Melton, Kevin Russell, Nikki Freed, Chris Barrozo, Jianguo Wu, Karl Rudnick, Anjali Desai, Emily Moradi, Duane J. Knize, David W. Robbins, James C. Hannis, Patina M. Harrell, Christian Massire, Thomas A. Hall, Yun Jiang, Raymond Ranken, Jared J. Drader, Neill White, John A. McNeil, Stanley T. Crooke, and Steven A. Hofstadler

# Rapid identification and strain-typing of respiratory pathogens for epidemic surveillance

David J. Ecker<sup>†\*</sup>, Rangarajan Sampath<sup>\*</sup>, Lawrence B. Blyn<sup>\*</sup>, Mark W. Eshoo<sup>\*</sup>, Cristina Ivy<sup>\*</sup>, Joseph A. Ecker<sup>\*</sup>, Brian Libby<sup>\*</sup>, Vivek Samant<sup>\*</sup>, Kristin A. Sannes-Lowery<sup>\*</sup>, Rachael E. Melton<sup>\*</sup>, Kevin Russell<sup>‡</sup>, Nikki Freed<sup>‡</sup>, Chris Barrozo<sup>‡</sup>, Jlanguo Wu<sup>‡</sup>, Karl Rudnick<sup>‡</sup>, Anjali Desai<sup>‡</sup>, Emily Moradi<sup>‡</sup>, Duane J. Knize<sup>‡</sup>, David W. Robbins<sup>‡</sup>, James C. Hannis<sup>\*</sup>, Patina M. Harrell<sup>\*</sup>, Christian Massire<sup>\*</sup>, Thomas A. Hall<sup>\*</sup>, Yun Jiang<sup>\*</sup>, Raymond Ranken<sup>\*</sup>, Jared J. Drader<sup>\*</sup>, Neill White<sup>\*</sup>, John A. McNeil<sup>\*</sup>, Stanley T. Crooke<sup>\*</sup>, and Steven A. Hofstadler<sup>\*</sup>

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Edited by Stanley Falkow, Stanford University, Stanford, CA, and approved April 11, 2005 (received for review December 31, 2004)

Epidemic respiratory infections are responsible for extensive morbidity and mortality within both military and civilian populations. We describe a high-throughput method to simultaneously identify and genotype species of bacteria from complex mixtures in respiratory samples. The process uses electrospray ionization mass spectrometry and base composition analysis of PCR amplification products from highly conserved genomic regions to identify and determine the relative quantity of pathogenic bacteria present in the sample. High-resolution genotyping of specific species is achieved by using additional primers targeted to highly variable regions of specific bacterial genomes. This method was used to examine samples taken from military recruits during respiratory disease outbreaks and for follow up surveillance at several military training facilities. Analysis of respiratory samples revealed high concentrations of pathogenic respiratory species, including *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pyogenes*. When *S. pyogenes* was identified in samples from the epidemic site, the identical genotype was found in almost all recruits. This analysis method will provide information fundamental to understanding the polymicrobial nature of explosive epidemics of respiratory disease.

genotyping | group A streptococci | infectious disease | *Streptococcus pyogenes* | pneumonia

Despite the prevalence of epidemic respiratory infections and their important impact on global human health, the molecular underpinnings of these conditions remain poorly understood. Epidemic respiratory infections can be caused by a wide variety of bacteria, including several species of *Streptococcus*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*, or viruses such as influenza virus, adenovirus, rhinovirus, or coronaviruses (1, 2). Although various culture methods, molecular techniques, and serologic diagnostic tests exist, for many epidemics the causative microorganism(s) are never determined. Furthermore, there has been no practical method for examining the broad bacterial ecology of respiratory infections to dissect the complex polymicrobial interactions that occur during explosive outbreaks of disease.

Group A streptococci (GAS), or *Streptococcus pyogenes*, is one of the most important organisms associated with respiratory infections because of its prevalence and its ability to cause severe disease with complications such as acute rheumatic fever and acute glomerulonephritis (3). The ability to simultaneously identify GAS and other bacteria and viruses in large numbers of samples would greatly facilitate our understanding of respiratory epidemics. It is also essential to follow the spread of specific virulent strains of GAS in populations and to distinguish virulent strains from avirulent streptococci (3).

Molecular methods have been developed to genotype GAS based on the sequence of the *emm* gene that encodes the M-protein virulence factor (4–6). More than 150 different *emm* types have

been defined and correlated with phenotypic properties of thousands of GAS isolates by using this molecular classification ([www.cdc.gov/ncidod/biotech/strep/strepindex.html](http://www.cdc.gov/ncidod/biotech/strep/strepindex.html)) (7). Recently, a strategy known as multilocus sequence typing (MLST) was developed to determine the molecular epidemiology of GAS and other bacterial pathogens. The results from MLST are highly concordant with several other typing methods (8).

Although MLST provides detailed analysis of isolated GAS strains, it provides no information about the other respiratory microbes that may participate in the pathology. We now report a technique that rapidly identifies multiple respiratory microorganisms simultaneously in a quantitative fashion. This allows for broad microbial population analysis and strain tracking of an ongoing, geographically dispersed epidemic on a large scale. We specifically identified the bacterial pathogens present during a respiratory disease outbreak at a military training camp (9), characterized the GAS strain-genotype, and analyzed the spread to other military facilities.

## Materials and Methods

Selected isolates used in this research from the Naval Health Research Center were collected in compliance with all applicable federal regulations governing the protection of human subjects in research under approved protocol NHRC.2001.0008.

**Primer Selection.** Broad-range PCR primers for mass spectrometry analysis were designed to target conserved regions of bacterial ribosomal DNA genes (16S and 23S) and genes encoding housekeeping proteins common to all bacteria (Table 1). Primers for genotyping GAS using mass spectrometric analysis were designed to target sequences from each of the seven housekeeping genes used in MLST. The nucleotide sequences for these genes from 212 isolates of GAS (78 distinct *emm* types) were obtained from [www.mlst.net](http://www.mlst.net). These correspond to the 100 different allelic profiles referred to by Enright *et al.* as ST1–ST100 (8). Twenty-four primer pairs were designed and validated against *S. pyogenes*. A final subset of six primer pairs (sequences are shown in Table 4, which is published as supporting information on the PNAS web site) was chosen based on a theoretical calculation of minimal number of primer pairs that maximized resolution between *emm* types.

**Mass Spectrometry and MLST.** After amplification, 15  $\mu$ L aliquots of each PCR were desalted and purified by using a weak anion exchange protocol as described (10). Accurate-mass ( $\pm 1$  ppm),

This paper was submitted directly (Track II) to the PNAS office.

Freely available online through the PNAS open access option.

Abbreviations: GAS, group A streptococci; MLST, multilocus sequence typing; ESI-MS, electrospray ionization mass spectrometry.

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**Table 1. Broad-range and *S. pyogenes*-specific genotyping primer targets and scope of coverage**

Primer #	Gene target	Bacterial target	Primer specificity
<b>Broad surveillance primers</b>			
348, 347; 348, 381	16S rDNA	ALL	Broadly conserved ribosomal genes
348, 360	23S rDNA		
<b><i>Streptococcus pyogenes</i> genotyping primers</b>			
358, 349	Ribosomal protein L2 (pL2)	Proteobacteria (γ, β, α), Clostridia, Firmicutes	

Sequences of the primers are provided in Table 4. Primer coverage of bacterial phylogeny is depicted pictorially in Fig. 4. The locations of the primers targeting ribosomal sequences are depicted mapped to the rRNA structures in Fig. 2 (16S) and 3 (23S).

high-resolution ( $M/\Delta M > 100,000$  full-width half maximal) mass spectra were acquired for each sample by using high-throughput electrospray ionization mass spectrometry (ESI-MS) protocols as described (11, 12). For each sample, 1.5  $\mu$ l of analyte solution was consumed during the 60-s spectral acquisition. Raw mass spectra were converted to monoisotopic molecular masses. Unambiguous base compositions were derived from the exact mass measurements (13). Quantitative results are obtained by comparing the peak heights with an internal PCR calibration standard present in every PCR well at 500 molecules for the ribosomal DNA-targeted primers and 100 molecules for the protein-encoding gene targets (11). GAS isolates were analyzed by using *emm* gene-specific PCR as described (4, 14). MLST analysis was performed as described (8).

## Results and Discussion

**Broad Surveillance, Identification, and Rapid Strain-Genotyping of Bacterial Pathogens.** To begin to decipher the polymicrobial dynamics that underlie epidemic outbreaks of respiratory disease, it would be valuable to analyze respiratory samples for a broad range of bacterial pathogens simultaneously and to obtain high-resolution strain-genotyping information on specific species. We have developed a rapid, high-throughput molecular method to achieve these objectives and have tested it on samples obtained from respiratory disease outbreaks associated with *S. pyogenes* in military training facilities (9). The experimental methodology is based on analysis of multiple PCR amplicons using PCR/ESI-MS to determine base compositions of complex mixtures of amplicons (11, 13). High-resolution genotyping of specific bacterial species, in this case *S.*

*pyogenes*, was accomplished by analyzing the samples with species-specific primers that interrogate regions of high intraspecies variability to distinguish closely related strains.

To measure the broad landscape of bacteria present in respiratory samples, a set of 16 broad-range surveillance primers was used that allow PCR amplification and quantitative identification of many different bacterial pathogens and respiratory commensal flora. Gene targets of these primers are listed in Table 1, and sequences are shown in Table 4. The surveillance primers were chosen by computational analysis of sequence alignments of the ribosomal DNA operons and 160 broadly conserved protein-encoding housekeeping genes. The ribosomal DNA-targeted primers have the broadest range of bacterial coverage. For example, the four primer pairs targeted to 16S ribosomal DNA match, on average, 98% of the bacterial sequences in the Ribosomal Database Project (<http://rdp.cme.msu.edu>) allowing for two to three mispairings under permissive PCR cycling conditions. The sites of hybridization and the sequence conservation in these regions are shown on the ribosomal RNA structures in Figs. 2 and 3, which are published as supporting information on the PNAS web site. The primers targeted to protein-encoding housekeeping genes have breadth of coverage at the level of major bacterial subdivisions (e.g., beta proteobacteria, bacilli); their specificity is described in Table 1 and graphically depicted in Fig. 4, which is published as supporting information on the PNAS web site.

Analysis of the amplified regions from major respiratory pathogens showed that the base compositions of these regions unambiguously distinguished all recognized respiratory pathogens from each other and from normal flora, including closely related species of *Streptococci* and *Staphylococci* (base compositions are listed in Table 5, which is published as supporting information on the PNAS web site). Although any single primer target region might have an overlap of base compositions with another species, combined information from multiple primer pairs provided unambiguous organism-specific signatures for all major respiratory pathogens. For example, *S. pyogenes* and *Streptococcus pneumoniae* have target regions that are amplified by 9 and 10 of the surveillance primers, respectively. The base compositions of these two species are identical in only one target region, and differ in all remaining target regions by up to four base substitutions per region. We confirmed the resolving power of the target regions by determining the base compositions of 120 isolates of respiratory pathogens representing 70 different bacterial species (data not shown). The results showed that the observed variations (usually one or two base substitutions in the amplified region) in base composition amongst multiple isolates of the same species did not prevent correct identification of major pathogenic species.

For high-resolution strain genotyping, we designed a strategy to generate strain-specific signatures that follows the rationale of MLST. We constructed an alignment of concatenated alleles of the seven MLST housekeeping genes from each of 212 previously *emm*-typed strains (8) and determined the number and location of the primer pairs that would maximize strain discrimination. An initial set of 24 primer pairs was selected that would amplify regions covering >97% of the nucleotide variation in the MLST sequencing targets. We then determined how much strain discrimination could be achieved from a smaller set of primers. Performance calculations for different possible combinations of primer subsets showed that six pairs of primers allowed discrimination at the individual *emm*-type level of >75% of all of the *emm* types listed by Enright *et al.* (8), whereas the remaining 25% clustered into groups of two or more *emm* types (see Fig. 5, which is published as supporting information on the PNAS web site, for details). This degree of resolution was considered sufficient for applications such as tracking the clonal expansion of a particular strain type during a specific epidemic. However, if complete *emm* typing is required, 12 primer pairs can be used to completely resolve all *emm* types.

Table 2. Base composition analysis of GAS samples

	Row #	# of Instances	EMM-type determination			Location	Date	Base Compositions					
			emm-type by PCR/ESI-MS	emm-gene sequencing	MLST			murI	mutS	xpt	yqil	gkl	gtr
Cultured Samples	1	48	3	3	M3	MCRD San Diego	2002	A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	2	2	6	6	M6			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	3	1	28	28	M28			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	4	15	3	ND	ND	NHRC San Diego-Archive	2003	A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	5	6	3	3	M3			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	6	3	5,58	5	M5			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	7	6	6	6	M6			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	8	1	11	11	ND			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	9	3	12	12	ND			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	10	1	22	22	M22			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	11	3	25,75	75	ND			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	12	4	44/61,82,9	44/61	M44/61			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	13	2	53,91	91	M91			A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	14	1	2	2		R. Leonard Wood	2003	A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	15	2	3	3				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	16	1	4	4				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	17	1	6	6				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	18	11	25 or 75	75				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	19	1	25,75, 33, 34, 52, 84	75				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	20	1	44/61 or 82 or 9	44/61				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	21	2	5 or 58	5		R. Sili	2003	A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	22	3	1	1				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	23	2	3	3				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	24	1	4	4				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	25	1	28	28				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	26	1	3	3				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	27	1	4	4		R. Benning	2003	A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	28	3	6	6				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	29	1	11	11				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	30	1	13	13				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	31	1	44/61 or 82 or 9	82				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	32	1	5 or 58	58				A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	33	1	78 or 89	89		Lackland AFB	2003	A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	34	2	5 or 58					A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	35	1	2					A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	36	1	81 or 90					A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	37	1	78					A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	38	3*	No detection					A40 G24 C20 T34	A40 G29 C19 T31	A40 G29 C19 T31	A40 G29 C19 T31	A31 G35 C17 T33	
	39	7	3	ND	ND	MCRD San Diego	2002	No detection	No detection	No detection	No detection	No detection	No detection
Swabs	40	1	3	ND	ND			No detection	No detection	No detection	No detection	No detection	No detection
	41	1	3	ND	ND			No detection	No detection	No detection	No detection	No detection	No detection
	42	1	3	ND	ND			No detection	No detection	No detection	No detection	No detection	No detection
	43	2	3	ND	ND			No detection	No detection	No detection	No detection	No detection	No detection
	44	3	No detection	ND	ND			No detection	No detection	No detection	No detection	No detection	No detection

Base compositions from each primer target site are color-coded so that each unique base composition has its own color. ND, no data.

\*Samples were determined to be GAS-negative by independent culture techniques.

**Identification and Strain Genotyping of GAS Isolates.** Four sets of throat samples taken from recruits at different military facilities were examined. The first set was collected at a military training center in 2002 during one of the most severe outbreaks of pneumonia associated with GAS in the U.S. since 1968 (9). Throat swabs were taken from both healthy and hospitalized recruits and plated for selection of putative GAS colonies. A second set of 15 original patient specimens was taken during the height of this disease outbreak. The third set were historical samples from disease outbreaks at this and other military training facilities during previous years. The fourth set of samples was collected from five geographically separated military facilities in the continental U.S. in the winter immediately after the severe 2002 outbreak.

Colonies isolated from GAS selective media from all four collection periods were analyzed with the broad surveillance primers and GAS genotyping primers (Table 1). When the surveillance primers were used, all samples showed base compositions that precisely matched the four completely sequenced strains of *S. pyogenes* (15–20). The results of the base composition analysis with genotyping primer pairs for samples from all four collection periods are compared to results from 5'-*emm* gene sequencing and the MLST gene sequencing methods in Table 2. When only these six primer pairs were used, some of the samples could not be resolved to a unique *emm* type. However, base composition analysis showed identification consistent with (either uniquely or as a member of a

small set) 5'-*emm* gene sequencing or the MLST sequencing method.

**Genotyping GAS Isolated from the 2002 Epidemic and Testing of Original Patient Specimens.** Of the 51 samples taken during the peak of the November/December 2002 epidemic (Table 2, rows 1–3), all but three had identical base compositions and corresponded to *emm*3, a GAS genotype previously associated with high respiratory virulence (17, 19). The three outliers (Table 2, rows 2 and 3) were samples from healthy individuals and probably represent nonepidemic strains harbored by asymptomatic carriers. Archived samples (Table 2, rows 5–13) from historical collections showed a much greater heterogeneity of composition signatures and *emm* types, as would be expected for different epidemics occurring at different times and places.

During the peak of the 2002 outbreak, duplicate throat swabs were taken from military recruits who were not overtly symptomatic but who were living and training in the same community. One of the paired swabs was used to isolate GAS colonies on selective media and the other swab was analyzed directly. Fifteen of the paired swabs that showed at least one colony on GAS-selective media were selected for further study. When the surveillance primers were used, all 15 of these GAS isolates showed base compositions identical to the sequenced GAS genomes, as was observed with all previous GAS isolates in this study. The six



GAS-specific genotyping primers indicated that all 15 samples had the same GAS genotype (Table 2, row 4), corresponding to *emm3*, the identical signature obtained from the symptomatic individuals in this outbreak, consistent with the clonal expansion of a single genotype.

The duplicate swabs were analyzed without culture by using the 16 broad surveillance and six GAS-specific genotyping primers. Analysis using the surveillance primers revealed that these samples had a mixture of microbes, as might be expected from a complex sample (Table 3). Of the 15 samples, six showed evidence of GAS using the broad surveillance primers, and seven showed positive detection using all six genotyping primers (Tables 3 and 2, row 39). Of the remaining eight samples, five were positive with two to four genotyping primers (Table 2, rows 40–43) and three of the samples were negative with all six genotyping primers (Table 2, row 44). These results suggest that GAS was present in these samples at widely varied concentrations.

In addition to GAS, other potentially pathogenic organisms were identified. In an exemplary sample (Table 3, sample 5), GAS was identified along with strong signals consistent with *N. meningitidis* and *H. influenzae* (Fig. 1). The 16 surveillance primers have a varying degree of breadth in their coverage. The six primers that target 16S and 23S ribosomal DNA were designed to amplify all bacteria from the major divisions (Table 1). Mass spectral analysis of the products from primers that target 16S and 23S ribosomal DNA (Fig. 1 Upper Right and Lower Left) showed that the dominant signals were from *H. influenzae*, *N. meningitidis*, and *S. pyogenes* (GAS) present in a ratio of  $\approx 20:5:4$  as determined by comparison of peak heights with that of internal PCR calibration standards for several of the primers. In contrast to the primers that amplify ribosomal DNA genes, the primers that target genes encoding housekeeping proteins were designed to provide coverage of specific divisions of bacteria. For example, primer pair 356 targets the *rplB* gene (Table 1) and primarily amplifies the bacterial classes Bacilli (which includes *S. pyogenes*) and Clostridia, but does not amplify Proteobacteria such as *N. meningitidis* and *H. influenzae*. Analysis of the spectrum from this primer set shows *S. pyogenes* as the only major product (Fig. 1 Lower Right). As expected, primers targeted to the proteobacterial species identified *N. meningitidis* (corroborating evidence for the simultaneous circulation of *N. meningitidis* during this epidemic was obtained from culture data from a hospitalized patient who subsequently died from pneumonia during this period; K. Russell, unpublished data) and *H. influenzae*, but not *S. pyogenes* (data not shown). Although base compositions detected from some of the surveillance primers are consistent with more than one organism, the collective data from the 16 surveillance primers unambiguously identified these three bacterial species as responsible for the bulk of the bacterial load in this throat swab. Thus, using this surveillance panel of primers, it is possible to identify the major bacterial components of a complex sample and to determine their approximate concentrations.

To corroborate the results of mass spectrometry analysis of PCR products obtained by using the surveillance primer set, we analyzed two of the samples by broad-range priming followed by cloning and sequencing (21). The results from sequencing  $\approx 700$  nucleotides of 16S ribosomal DNA were in good agreement with the mass spectrometry analysis with respect to both identification of species and the relative abundance for the organisms that constituted  $> 5\%$  of the total microbial load in the sample (details in Table 6, which is published as supporting information on the PNAS web site). However, cloning and sequencing indicated the presence of additional species of bacteria not identified by PCR/ESI-MS. For example, based on sequencing, 5% of the bacterial load in sample 5 (Table 3) was comprised of *Corynebacterium fastidiosum*. Retrospective analysis of the mass spectra revealed peaks that were consistent with the presence of this organism, but the peak heights across the surveillance primer set were insufficient to make a positive identification. Thus, broad-range primer analysis will be

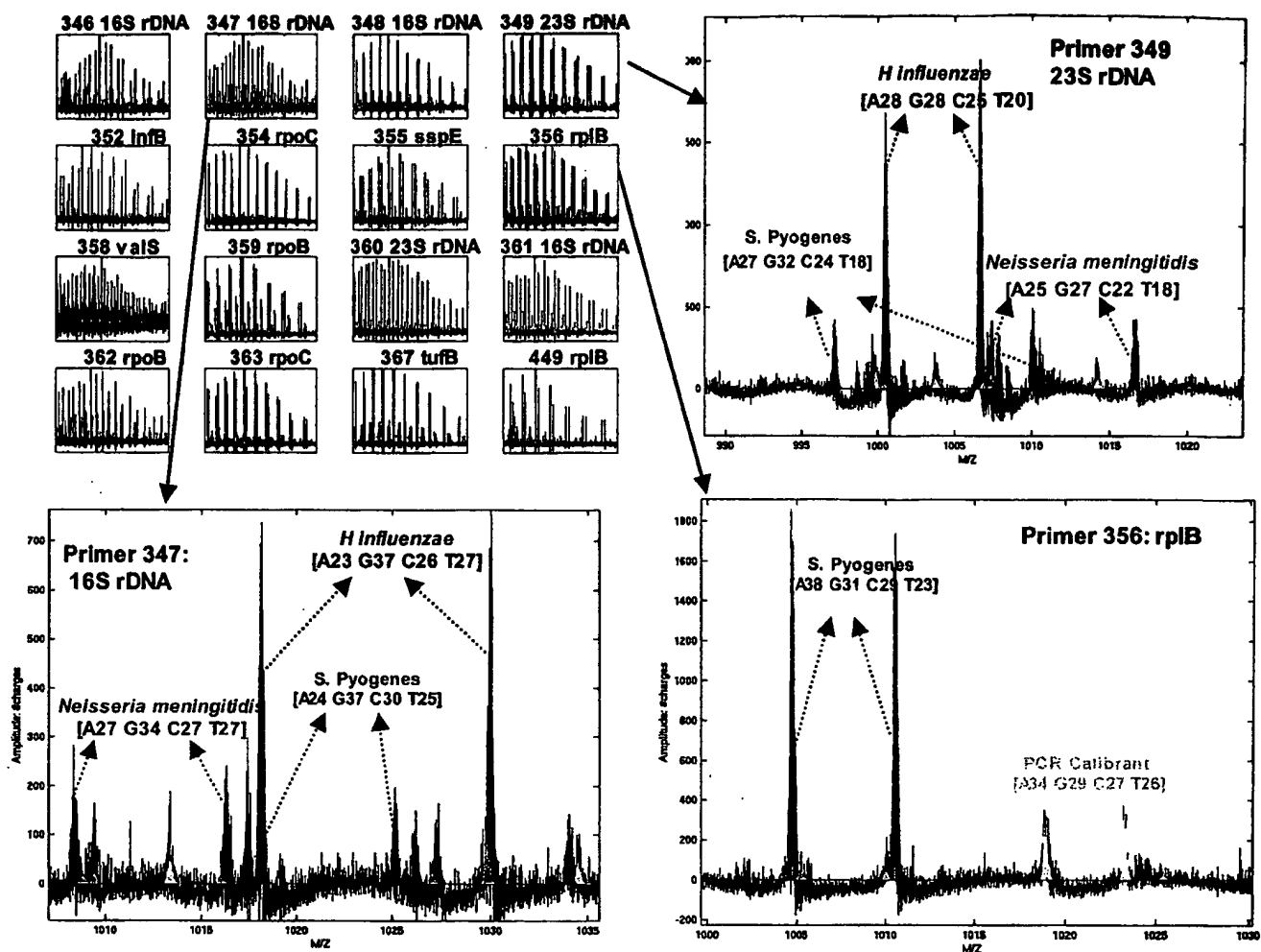
Table 3. Analysis of 15 throat swabs

No.	Organisms identified	Relative ratios	Positive by genotyping
1	<i>H. influenzae</i>	5	6
	<i>S. pyogenes</i>	1	
2	<i>N. meningitidis</i>	14	0
	<i>H. influenzae</i>	10	
3	<i>H. influenzae</i>	2	6
	<i>S. pyogenes</i>	1	
4	<i>H. influenzae</i>	NA	0
5	<i>H. influenzae</i>	20	6
	<i>N. meningitidis</i>	5	
6	<i>S. pyogenes</i>	4	0
	<i>H. influenzae</i>	5	
7	<i>C. pseudodiphtheriticum</i>	1	6
	<i>S. pyogenes</i>	NA	
8	<i>S. epidermidis</i>	NA	3
9	<i>N. meningitidis</i>	7	6
	<i>S. pyogenes</i>	1	
10	<i>H. influenzae</i>	2	2
	<i>S. pneumoniae</i>	1	
11	<i>N. meningitidis</i>	$>20$	6
	<i>S. gordonii</i>	1	
12	<i>M. catarrhalis</i>	2	0
	<i>H. influenzae</i>	1	
13	<i>N. meningitidis</i>	NA	0
14	<i>S. pyogenes</i>	5	6
	<i>S. aureus</i>	1	
15	<i>N. meningitidis</i>	$>20$	4
	<i>S. mitis</i>	1	

less sensitive on an absolute scale for a low abundance organism than species-specific primers that do not have to compete for PCR resources with multiple microbes (see sensitivity measurements below). The other sample that was analyzed by both PCR/ESI-MS and sequencing (Table 3, sample 14) contained *S. pyogenes* and *Staphylococcus aureus* in a ratio of  $\approx 5:1$  as determined by PCR/ESI-MS and about 3.5:1 by sequencing.

It is interesting that the 15 throat swabs from military recruits contained a relatively small set of microbes in high abundance (Table 3). The most common were *H. influenzae*, *N. meningitidis*, and *S. pyogenes*; *S. epidermidis*, *M. catarrhalis*, *C. pseudodiphtheriticum*, and *S. aureus* were present in fewer samples. We also analyzed an equal number of samples from healthy volunteers in the same fashion and did not observe the same pattern of microbes (samples were taken from 23 healthy volunteers from each of three different geographic locations, not from military training settings). Healthy volunteers showed a flora dominated by multiple, commensal non- $\beta$ -hemolytic Streptococcal species, including viridans group streptococci (*Streptococcus parasanguinis*, *Streptococcus vestibularis*, *Streptococcus mitis*, *Streptococcus oralis*, and *S. pneumoniae*; data not shown). Thus, the military recruits in the midst of a respiratory disease outbreak had a dramatically different microbial population than that experienced by the general population in the absence of epidemic disease.

**Genotyping GAS Isolated from Geographically Separated Military Facilities in 2003.** After the 2002 epidemic associated with a virulent *emm3* strain, we surveyed respiratory disease outbreaks at other military facilities. It was possible that the virulent genotype from the epidemic might have spread to these locations later in the winter season. GAS isolated from patients with respiratory disease was examined by base composition analysis and by *emm*-gene sequencing. The results (Table 2, rows 14–33) showed concordance between base composition analysis and *emm* gene sequencing. One or two samples from each location had an *emm3* genotype. However, the distribution of GAS types at these locations showed a pattern significantly different from the original epidemic, suggesting that



**Fig. 1.** Mass spectra from DNA amplified from a throat swab (Table 3, sample 5) using each of the 16 surveillance primers (Table 1). (Upper Left) Thumbnail spectra from 16 primers. Each of the PCR wells was calibrated by using an internal standard identical to the bacterial target sequence except for a 2- to 5-nt internal deletion (calibrant peaks are shown in yellow). (Upper Right) Spectrum from a primer pair that targets 23S rDNA. The paired peaks correspond to the sense and antisense strands of the PCR amplicon that are separated under conditions of ionization. The peaks are labeled with base composition of the amplicons and the organism that matches the composition. (Lower Left) Spectrum from a primer pair that targets 16S rDNA. (Lower Right) Spectrum from a primer pair that targets the *Bacilli*, but not the *Proteobacteria*.

the epidemic strain was not dominating the population of GAS at other locations.

Throat swabs from eight individuals showing respiratory symptoms were obtained and analyzed after culture and directly from the swab. Five of the eight patients tested positive for GAS by culture on selective media (Table 2, rows 34–38). Samples that were culture-positive were also GAS-positive by base composition analysis when analyzed directly from the swab, whereas the three culture-negative samples were also negative by PCR/ESI-MS.

**Sensitivity, Dynamic Range, and Reproducibility.** To evaluate the limit of detection, serial 2-fold dilutions of known amounts of genomic DNA isolated from *S. pyogenes* were added to water or genomic material isolated from throat swabs from healthy volunteers. In water, both the broad surveillance and genotyping primers reliably detected as few as 15 genome copies of *S. pyogenes* per PCR well (data not shown). In the presence of normal throat flora, the *S. pyogenes* genotyping primers, which do not competitively amplify commensal streptococcal species, had the same sensitivity as in water. However, the broad surveillance primers lost sensitivity for *S. pyogenes* in the presence of floral DNA because of PCR

competition with commensal streptococcal organisms. The limit of detection was  $\approx 2,500$  genomic copies per well in the presence of the average amount of normal flora taken from a throat swab (pooled from 15 volunteers and divided to one swab-equivalent). On the other hand, when *K. pneumoniae* and *B. anthracis* were spiked into normal throat flora, the limit of detection was  $\approx 10$ –30 genome copies per well for both organisms. The difference in sensitivity for *S. pyogenes* vs. *K. pneumoniae* and *B. anthracis* can be attributed to the predominance of streptococcal organisms in normal flora. Although all of the surveillance primers that amplify *S. pyogenes* also competitively amplify commensal streptococcal species, several of the surveillance primers (in particular those that target genes encoding housekeeping proteins) amplify *K. pneumoniae* and *B. anthracis* and exclude the commensal streptococci. Thus, the lower limit of detection for a particular organism is not absolute, but varies with the level and nature of competing DNA and the coverage of the surveillance primers (see dynamic range experiment below). This is uniquely problematic for *S. pyogenes* in a throat flora background, which is dominated by commensal streptococcal species. To detect low levels of *S. pyogenes* in the presence of throat flora, one or more of the *S. pyogenes*-specific genotyping primers

that do not amplify commensal streptococcal species would be necessary.

To determine the dynamic range and linearity of competitive PCR/ESI-MS, we mixed three organisms in varying relative ratios ranging from 10 to 10,000 and analyzed them by using the surveillance primer set. The results (Fig. 6, which is published as supporting information on the PNAS web site) showed a dynamic range of at least 1,000:1, where one organism could be detected in the presence of a 1,000-fold excess of one or two other organisms. The deviation from linearity at 45 cycles of PCR over a 1,000:1 dynamic range was  $\pm 60\%$ . The dynamic range would vary somewhat for different mixtures of organisms, depending on their coverage by the surveillance primers. Having a mix of primers with varying breadth in specificities effectively expands the overall dynamic range of the system while assuring that the major bacterial components of a mixture are identified.

To assess swab-to-swab variation, we analyzed duplicate throat swabs from 23 healthy volunteers. The composition of the bacterial flora varied somewhat from individual to individual, but the replicate swabs from the same donor showed virtually identical profiles (Fig. 7, which is published as supporting information on the PNAS web site). The bacteria from these duplicate swabs were all dominated by commensal *Streptococcus* spp., as expected for normal throat flora from healthy donors (21).

## Conclusions

In both developing and developed nations, the leading cause of death by a wide margin is acute respiratory disease (22–24). However, the underlying microbial ecology and the polymicrobial interactions that mediate explosive epidemics remains poorly understood. We have developed a strategy to simultaneously survey respiratory samples for the presence of many different pathogenic agents and to provide high-resolution strain genotyping for selected species of bacteria. Using a set of surveillance primers targeted to

broadly conserved regions of bacterial genomes, PCR amplicons were generated and analyzed by ESI-MS, and the identity and relative quantity of microorganisms was determined by using the base compositions of the amplicons. This method allows rapid detection of the abundant microbial flora present in a complex sample. To track a particular bacterial strain that may be the driving force of an epidemic, high-resolution genotyping capability is required. This is accomplished by the use of species-specific primers that target regions of high variation.

In this study, we analyzed four sets of respiratory samples from military settings. Military recruits live in close quarters and are subject to intense physical and vocal stress as a normal part of training. Analysis of respiratory samples from military recruits living in a training community where a high amount of respiratory disease was present showed high concentrations of one or several pathogenic respiratory bacteria, including GAS, *H. influenzae*, and *N. meningitidis*. From the epidemic site, the identical GAS genotype was identified in almost all recruits. The respiratory flora present in these recruits was not found in healthy controls.

We have developed a rapid, high-throughput, and cost-effective method for surveying large numbers of samples that provides both a broad view of the bacterial organisms present and a high-resolution genotype of selected species. The PCR/ESI-MS analysis of 96 samples with all surveillance primers takes  $\sim 19$  h, and has sufficient speed and throughput to be useful in tracking of an ongoing epidemic. Although this work focused on identification of bacteria, with detailed strain genotyping of GAS, the PCR/ESI-MS method described here can be extended to broad groups of viruses (25), fungi, and pathogenic protozoa. We envision using this methodology to enhance our understanding of the fundamental nature of explosive epidemics of respiratory disease.

We acknowledge Dr. Jackie Wyatt for editorial assistance and Defense Advanced Research Planning Agency for financial support.

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## EXHIBIT B

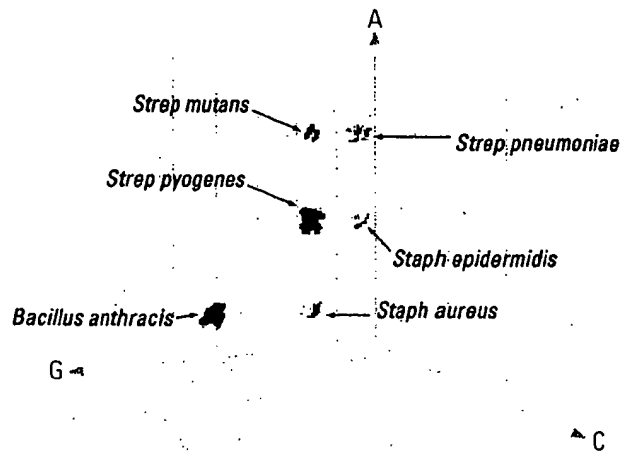
## Detecting newly emerging pathogens by MS

Suppose that thousands of people in Washington, DC, are suddenly falling ill. Severe body aches and mysterious lesions are the main symptoms. Hospitals are inundated with patients. Technicians are running lab tests as fast as they can, but results from culturing can take as many as seven days, and the tests are coming back negative for all known organisms. PCR-based tests, although faster, are also failing to detect the new pathogen. What can be done?

Steven Hofstadler and colleagues at Ibis Therapeutics and Science Applications International Corp. are working on a solution just in case such a scenario does take place. With funding from the U.S. Department of Defense's Defense Advanced Research Projects Agency, they have developed Triangulation Identification for Genetic Evaluation of Risk (TIGER)—a new strategy for identifying both known and previously uncharacterized pathogens. TIGER can be used to identify a wide range of organisms, such as viruses, bacteria, fungi, and parasitic protozoa.

The conventional methods of culturing swabbed samples and using PCR primers to amplify species-specific regions of organisms' genomes produce true/false results, and many iterations must be run, says Hofstadler. "TIGER is one thorough test that answers the question, 'What is in my sample?' It's a big essay."

According to Hofstadler, TIGER's power comes from the use of broad-range primers. "A broad-range primer is one that lands and hybridizes to highly conserved regions of the genome but that flanks variable regions that have large differences in base composition," he explains. For example, although a particular set of primers may bind to the genomes of all *Streptococcus* species, the actual PCR products will contain different amounts of As, Gs, Cs, and Ts, depending on the species. The primers are also designed so that they all work well



A base composition plot for bacterial species.

under identical conditions. Therefore, samples can be loaded onto 96- or 384-well plates for high-throughput PCR. Following PCR, the nucleic acid products are analyzed by MS.

Sequence information is not obtained by the method. Instead, each PCR product has a characteristic number of nucleotide bases and a unique  $m/z$  value, and these data are used to determine the identity of the pathogen. If an unexpected composition is discovered, the researchers map the new signature in 3-D or 4-D space along axes representing each nucleotide. Base composition signatures for other organisms are also plotted. From this, a phylogenetic tree of relatedness can be constructed.

TIGER can detect mixtures of organisms in the same sample. For example, Hofstadler says that by using TIGER, his group identified the SARS virus as a new member of the coronavirus family. When he and his co-workers mixed SARS with two other coronaviruses, they clearly observed six peaks on the mass spectrum, two for each species.

In December 2002, the U.S. Naval Health Research Center (NHRC) contacted Hofstadler and his team to identify the pathogen responsible for an out-

break among Marine recruits. The researchers confirmed that *Streptococcus pyogenes* was the culprit. "But the next thing they wanted to know was the Emm-type, the 'flavor' of *Strep*," says Hofstadler. Sequencing is typically used for this analysis, but his group used TIGER to obtain base composition tags instead. It took 6 min to type

each sample by TIGER. "We blew through these and rapidly had a bunch of Emm-types that we thought were correct, and this was in December," he says. "Then, in March, when [NHRC] finally got their sequencing results back and compiled, we sat down and agreed that we agreed."

In addition to running the method through its paces, the researchers are also working on integrating all the components into a laboratory-based system, which will be called TIGER 2.0. The Ibis group is already developing the next version of TIGER, which is being designed as a bench-top system suitable in size for clinical diagnostics laboratories.

Biowarfare, infectious disease epidemics, food contamination, and human forensics are just a few of the applications that Hofstadler foresees for the new method. His group is collaborating with many scientists in several government agencies, and interest is strong, he says. TIGER has a good track record, too. "We've analyzed over 20 million liters of air with TIGER," he says. "We've never false alarmed on a threat agent nor have we ever missed detecting an agent that we've intentionally spiked into the air sample."

COURTESY OF STEVEN HOFSTADLER, IBIS THERAPEUTICS

## EXHIBIT C

# The Universal Biosensor

A drug company tries to make a detector that can find nearly any biopathogen By GARY STIX

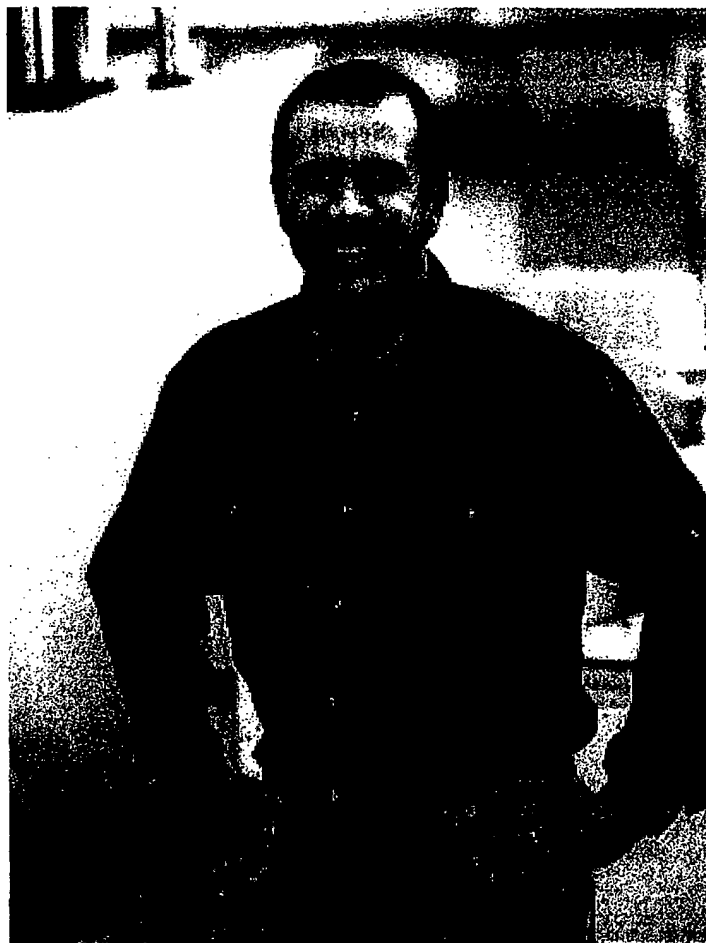
**Chance is often** the best inventor. Isis Pharmaceuticals never set out to become a maker of sensors for biological weapons. The company, based in Carlsbad, Calif., is best known for its work in developing anti-

sense therapies, the use of small pieces of DNA-like molecules that bind to messenger RNA (a copy of a gene) to block synthesis of an encoded protein. Its research led to the formation of a division called Ibis Therapeutics, which develops chemicals other than DNA that would interfere with RNA.

Along the way, Ibis discovered a method of screening pathogens that might lead to a universal detector for biological weapons—even perhaps nefarious, as yet to be invented bioengineered strains of pathogens. The road to a universal biosensor began in the mid-1990s, when Ibis started looking for chemicals with a low molecular weight that would bind to and block the activity of RNA, the same mechanism used by many antibiotics. The Defense Advanced Research Projects Agency (DARPA) funded some of the research because of its interest in finding new drugs to counter the microorganisms used in biowarfare. Conventional high-throughput screening—conducting a multitude of tests to measure the interaction of drug candidates with different enzymes—is ineffective for drugs that would work by binding to RNA. So Ibis began to explore the possibility of using mass spectrometry to determine when a small molecule binds to RNA.

The company refined a technique called electrospray ionization, as well as mass spectrometry, to extract RNA and the bound drug candidate from an aqueous solution intact and then suspend those molecules in a vacuum, where they can be weighed. As the methods proved themselves, Ibis president David J. Ecker came to the realization that pulling out the RNA alone, without the bound molecule, would provide the makings of an extraordinary sensing system.

After RNA from a cell is weighed with the spectrometer—each cell has multiple types of the molecule—these very precise measurements, accurate down to the mass of a few electrons, can be correlated with a database that contains information about RNA weights for a given pathogen. Each weight in the data-



INSPIRATION for Ibis Therapeutics's broad-scan biodeceptor came when company president David J. Ecker realized that a method used to screen for potential RNA-binding drugs might provide a means of looking for pathogens.

COURTESY OF ISIS PHARMACEUTICALS



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base table corresponds to the weight of the exact number of letters, or nucleotides, for a particular RNA. As long as information about the nucleotide composition is in the database, the system, called TIGER (triangulation identification for genetic evaluation of risks), can identify any bacterium, virus, fungus or protozoan. Before the RNA is weighed, another critical step is necessary: the poly-



**MICROBIAL SCALE:** The TIGER system uses a mass spectrometer to gauge the weight of a microorganism's RNA.

merase chain reaction must make copies of stretches of DNA or RNA that are found in all cellular organisms (or, for viruses, in whole families of them).

Six months before last year's anthrax attacks, Ibis and partner SAIC, a contract research house, received a \$10-million DARPA grant extending over two years to do a feasibility study for TIGER. The goal of the program is to develop a system that can detect the 1,500 or so agents known to infect humans. This approach differs fundamentally from the way other biotectors are designed. Most systems use an antibody or a piece of DNA as a probe to bind to a protein or nucleic acid in a pathogen. These tests are limited to detecting a small subset of the universe of pathogenic agents. And an antibody probe for, say, anthrax needs to make a match with the exact strain of the specific bacterium it is targeting.

COURTESY OF ISIS PHARMACEUTICALS

With TIGER, if information about a pathogen is not in its database—because it is a newly evolved strain or a specially bioengineered bug—the software can flag any genetic likeness it has with other microorganisms. “The database will say, ‘I’ve never seen this before, but it’s very similar to *Yersinia pestis* [plague],’” Ecker says. The detector would not, however, be able to pick up some genetic alterations of a microorganism—for instance, a gene for a toxin put in an otherwise harmless microbe.

Although biosensors were never part of Ibis’s business plan, about half of its 35 employees are now on the TIGER team.

If information about a pathogen is not in its database, TIGER might say, “I’ve never seen this before, but it’s very similar to the plague bacterium.”

Work at the company continues on sequencing the relevant genes to extract the needed RNA signatures for populating the databases—or obtaining this information from sequencing efforts under way worldwide. One of the biggest challenges the researchers still face is how to tell one piece of RNA from among thousands of specimens in a complex sample, such as a ball of dirt. “That requires very complex signal processing,” Ecker says. The problem that Ibis had encountered was one that radar engineers deal with constantly. In fact, this was the reason behind the collaboration with SAIC, which produced culture shock when Ibis’s molecular biologists began to work with SAIC’s radar engineers. “We spent the better part of a whole year figuring out how to communicate with each other,” Ecker remarks.

According to Ecker, it would have been easy to detect the anthrax in the letter sent to Senator Tom Daschle of South Dakota in October 2001, because the envelope contained no other biological material. Finding a small amount mixed in with other organic molecules is much harder; researchers are still laboring to improve the signal-processing capabili-

ties. The extent to which TIGER can read pathogen signatures in complex samples will determine how effective the technology is. “The question is how far can we ultimately push it,” Ecker says.

In April, Nobelist Joshua Lederberg, a scientific adviser to Ibis, hosted a conference at the Rockefeller University to explore ways in which various government agencies could adapt TIGER to their particular needs. If tests prove successful, Ecker foresees a detector eventually in every hospital, clinic and surveillance center, which could report back to a central monitoring site. How many of these systems would be deployed would

depend in part on society’s fear level about biowarfare—each of the mass spectrometers alone could cost \$200,000. “Although TIGER is an extremely powerful tool, it is a big, cumbersome and expensive machine. Plus, it does not give results in real time,” notes Rocco Casagrande, a biologist with Surface Logix, a drug-discovery company that has done work in biodetection [see “Technology against Terror,” by Rocco Casagrande; *SCIENTIFIC AMERICAN*, October].

Ecker’s optimism about the technology, though, extends beyond bioweapons. The detection system can be used to look not only for biopathogens but for any kind of disease-causing organism. Ecker believes that it could enable laboratories to forgo many of the time-consuming processes needed to determine if a particular microorganism is present—whether that bug is measles, anthrax or a newly emerging infectious disease. “If my vision holds, this could supersede a lot of what takes place in infectious microbiology,” he says. “There would be no need to culture things anymore.” Thus, a bioweapon sensor could become a universal disease sentinel.

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## EXHIBIT D

### THE EVOLVING FIELD OF BIODEFENCE: THERAPEUTIC DEVELOPMENTS AND DIAGNOSTICS

James C. Burnett\*, Erik A. Henchaft, Alan L. Schmaljohn† and Sina Bavari‡

**Abstract** | The threat of bioterrorism and the potential use of biological weapons against both military and civilian populations has become a major concern for governments around the world. For example, in 2001 anthrax-tainted letters resulted in several deaths, caused widespread public panic and exerted a heavy economic toll. If such a small-scale act of bioterrorism could have such a huge impact, then the effects of a large-scale attack would be catastrophic. This review covers recent progress in developing therapeutic countermeasures against, and diagnostics for, such agents.

#### BACILLUS ANTHRACIS

The causative agent of anthrax and a Gram-positive, spore-forming bacillus. This aerobic organism is non-motile, catalase positive and forms large, grey-white to white, non-haemolytic colonies on sheep blood agar plates.

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Microorganisms and toxins with the greatest potential for use as biological weapons have been categorized using the scale A–C by the Centers for Disease Control and Prevention (CDC). This review covers the discovery and challenges in the development of therapeutic countermeasures against select microorganisms and toxins from these categories. We also cover existing antibiotic treatments, and early detection and diagnostic strategies for intervening against these biothreat agents at a point in disease progression when the prognosis can still be influenced; and to guide the selection of the optimum therapeutic protocols. Furthermore, although a detailed review of vaccines for biothreat agents exceeds the scope of this manuscript, an important point to consider is that the described therapeutics will most likely be used in combination with vaccines, which possess the advantage of providing long-term immuno-protection.

#### Countering biological toxins

Research to identify/develop therapeutics against biological toxins falls into two categories: relatively large biological inhibitors, such as antibodies and decoy proteins; and small-molecule inhibitors (both peptidic and non-peptidic). The identification and development of therapeutics against anthrax toxin, botulinum neurotoxins, ricin toxin and staphylococcal enterotoxins are discussed. This section is limited mainly to

small-molecule inhibitors, and a brief review of antibody development and design against biotoxins is mentioned in TABLE 1.

**Anthrax toxin.** The toxin secreted by *BACILLUS ANTHRACIS*, ANTHRAX TOXIN (ATX), possesses the ability to impair innate and adaptive immune responses<sup>1–3</sup>, which in turn potentiates the bacterial infection. This suggests that inhibiting ATX activity is a viable therapeutic modality — blocking the actions of this toxin should provide the window of opportunity that is necessary for conventional antibiotics, in combination with the inherent immune response, to clear the bacterium well before deadly sepsis and toxic shock occur. FIGURE 1 shows how lethal toxin (LT, which comprises protective antigen (PA) + lethal factor (LF)), attacks cells. The potency of LT is shown in TABLE 2.

The action of ATX can be inhibited in several ways. One method would be to interfere with the furin-mediated cleavage of PA to its active form (PA<sub>63</sub>) following host-cell receptor binding<sup>4–7</sup>. To this end, hexa-D-arginine has been identified<sup>8</sup>, and has demonstrated the capacity to delay ATX toxemia *in vivo*<sup>9</sup>. Following this approach, a more potent nona-D-arginine has been generated<sup>10</sup>.

Non-functional (decoy) PA mutants that co-assemble with wild-type PA, and interfere with LF/oedema factor (EF) transport into the host-cell cytosol, have shown



# ANTHRAX TOXIN

A complex composed of three proteins: protective antigen (PA), lethal factor (LF) and oedema factor (EF).

# SNARE COMPLEX

A complex composed of SNAP25, VAMP (also referred to as synaptobrevin) and syntaxin that is involved in membrane fusion and the exocytosis of acetylcholine into neuromuscular junctions.

Table 1 | Antibodies that target biological toxins

Target	Source	Comments	References
Anthrax toxin	Human, humanized and murine	Many have shown protection against both anthrax lethal toxin and <i>Bacillus anthracis</i> .	180–185
Botulinum neurotoxins	Human, humanized and murine	Antibodies against all seven serotypes are needed. Some have been used in combination; affinity might be crucial for protection. Broad neutralizing antibodies are needed. Most antibodies are against the carboxyl end of the toxin; should explore other sites.	186–194
Ricin	Avian and murine	Have shown protection <i>in vivo</i> . Critical need for high affinity antibodies. Genetically inactivated ricin can be used as an antigen. Aerosolized ricin induces lung damage even in surviving subjects <sup>201</sup> . Adjunctive therapeutics are desired.	195–202
Staphylococcal enterotoxins	Human, avian and murine	Some have shown protection against aerosolized toxin, need an antibody with broad neutralizing activity against all staphylococcal enterotoxins.	62,203,204

promise<sup>11,12</sup>. Another method would involve interfering with PA–LF or PA–EF binding events. A polyvalent compound consisting of a polyacrylamide backbone substituted with multiple copies of a peptide (HTSTY-WWLDGAP) provides protection against LF<sup>13</sup>. Finally, identifying or generating molecules that bind within the PA heptamer pore, thereby blocking LF/EF release into the host-cell cytosol, is also a potential avenue for toxin inhibition. In anticipation of such research, Nguyen<sup>14</sup> has generated a structurally viable PA heptamer model that will be useful for future drug discovery.

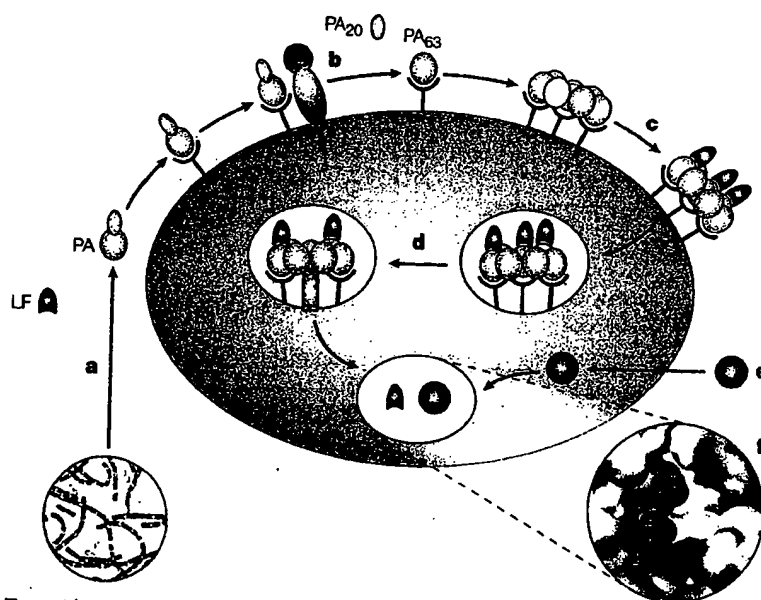


Figure 1 | A schematic of anthrax toxin (ATX) lethal factor cell entry. a | ATX is secreted by *Bacillus anthracis*. b | The inactive form of protective antigen (PA<sub>20</sub>) binds to a host-cell receptor, where it is cleaved by a furin-related protease, to give active PA<sub>63</sub>. c | PA<sub>63</sub> heptamerizes and can bind to either lethal factor (LF) or oedema factor (EF) (in this depiction the heptamer binds LF). d | The complex is endocytosed, and LF (as shown) or EF (not shown) translocates from the endosome into the host-cell cytosol. e | Therapeutics, in this example NSC 12155 (REF. 19), are being designed to enter intoxicated cells and inhibit the protease activity of LF. f | A surface depiction of NSC 12155 bound within the LF substrate-binding cleft is shown. The inhibitor carbons are green, nitrogens are blue and oxygens are red. The surface of LF is red for acidic surface, blue for basic surface, and white for neutral surface.

LF has been recognized as one of the main virulence components of *B. anthracis*. Consequently, there is much interest in identifying inhibitors of this metalloprotease. Several hydroxamate inhibitors of LF have been identified<sup>15,16</sup>, one of which, In-2-LF<sup>16</sup>, has a  $K_i = 1.0$  nM *in vitro*. By incorporating a metal-chelating moiety, a potent inhibitor MKARRKKVYP-NHOH ( $K_i = 0.0011$   $\mu$ M) was generated<sup>17,18</sup>. Using this information, additional peptidic inhibitors were identified<sup>17</sup> (TABLE 3). Panchal *et al.*<sup>19</sup> used a high-throughput assay to analyse the National Cancer Institute's (NCI's) Diversity Set. Several small (non-peptidic) molecules with drug-like properties were identified (TABLE 3; FIG. 2a). Some of these compounds were identified via subsequent three-dimensional database mining. On the basis of compounds identified during this study, a common pharmacophore for LF inhibition was generated that will provide a template for identifying new leads. The search for LF inhibitors has also prompted the application of less conventional technologies — for example, a mass spectrometry-based technique was used to identify the inhibitor DS-998 (TABLE 3)<sup>20</sup>. Finally, nature has proven once again to be a pharmaceutical treasure chest: natural products, including epigallocatechin-3-gallate ( $IC_{50} = 97$  nM), an isolate of green tea (TABLE 3)<sup>21</sup>, and aminoglycosides, including neomycin B ( $K_i = 7.0$  nM)<sup>22</sup>, are potent LF inhibitors.

Two notable inhibitors of the adenylate cyclase activity of EF were identified during a screen of the Available Chemical Directory database<sup>23</sup> (TABLE 4), whereas an active metabolite of adefovir dipivoxil (TABLE 4) was found to selectively inhibit EF with high affinity<sup>24</sup>.

**Botulinum neurotoxins.** Botulinum neurotoxins (BoNTs) are the most potent of the biological toxins (TABLE 2), are easily produced and can be delivered via an aerosol route<sup>25</sup>. There are seven BoNT serotypes (A–G), and each cleaves a specific component of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor SNARE complex. This cleavage impairs the release of acetylcholine, and can lead to deadly flaccid paralysis. The toxin is composed of a heavy chain

Table 2 | Comparative biological potency of biodefence toxins

Toxin	LD <sub>50</sub> (µg per kg)	Source
Botulinum toxin A	0.001	Bacterium
Tetanus toxin	0.002	Bacterium
Shiga toxin	0.002	Bacterium
Staphylococcal enterotoxin B	0.02*	Bacterium
Diphtheria toxin	0.1	Bacterium
Maitotoxin	0.1	Marine dinoflagellate
Ciguatoxin (P-CTX-1)	0.2	Marine dinoflagellate abrin plant
	0.7	
Batrachotoxin	2	Poison arrow frog
Ricin	3	Plant
Tetrodotoxin	8	Pufferfish
Saxitoxin	10	Marine dinoflagellate
Staphylococcal enterotoxin B	10 (aerosol nonhuman primates)	Bacterium
Anthrax lethal toxin	50*	Bacterium
Microcystin	50	Blue-green algae
Aconitine	100	Plant
T-2 toxin	1,200	Fungus

\*Predicted human aerosol. †Based on rat model of anthrax PA and LF toxicity. REF. 25 provides an excellent review on inhaled biological toxins. Table adapted from REF. 206.

#### RICIN TOXIN

Isolated from seeds of the castor plant (*Ricinus communis*), ricin toxin consists of a 32-kDa B chain that is linked by a disulphide bridge to a 32-kDa A chain (RTA)<sup>17,18</sup>. The B chain binds cell surfaces. Once inside the cell cytoplasm, RTA is released, and irreversibly depurinates the 28S rRNA, destroying the elongation-factor-binding site, and thereby disabling cellular protein synthesis<sup>17,18,19</sup>.

#### STAPHYLOCOCCAL ENTEROTOXINS

A large group of protein toxins that engage both major histocompatibility complex class II molecules on the surface of antigen-presenting cells and the variable (V)  $\beta$ -chain of a large subset of T-cell receptors.

(HC) that targets gangliosidic receptors on nerve terminals, forms a low-pH endosome and translocates the light chain (LC) into the nerve cytosol<sup>26–28</sup>. The LC acts as a zinc metalloprotease, and is responsible for SNARE protein cleavage<sup>29–31</sup>. The HC and the LC therefore provide two viable targets for neutralizing this toxin. The vast majority of research to identify BoNT therapeutics has focused on serotypes A and B. With regard to inhibiting HC activity, Deshpande *et al.*<sup>32</sup> and Sheridan *et al.*<sup>33</sup> have proposed that several antimalarial compounds, which delay muscle paralysis following BoNT serotype A (BoNT/A) challenge, act by interfering with the acidity of the toxin-mediated endosome. In addition, Eswaramoorthy *et al.*<sup>34</sup> have generated a co-crystal structure of doxorubicin bound within the BoNT serotype B (BoNT/B) HC ganglioside-binding site. Such inhibitors would interfere with the ability of the toxin to bind to its neuronal receptor.

LC inhibitors would be crucial to rescuing nerve activity after toxin internalization. In the search for such therapeutics, a number of short 'hinge' peptide inhibitors of the BoNT/A LC have been described<sup>35</sup>. However, the structures of these hinge peptides were not deconvoluted from the test mixtures. Using a substrate-to-inhibitor strategy, Schmidt and co-workers<sup>36–39</sup> generated potent inhibitors of the BoNT/A LC (TABLE 5). Subsequently, a similar strategy was used by Sukonpan *et al.*<sup>40</sup> to identify additional peptidic inhibitors. In a recent study<sup>41</sup>, small (non-peptidic and non-chelating) drug-like molecules that inhibit the BoNT/A LC were discovered (TABLE 5). Two of the most potent inhibitors, michellamine B and Q2-15 (FIG. 2b), are shown in TABLE 5. On the basis of the identified inhibitors and

molecular docking using LCs obtained from available X-ray crystal structures<sup>42,43</sup>, a pharmacophore for BoNT/A LC inhibition was generated<sup>41</sup> that will be of value for ongoing drug discovery. Furthermore, Breidenbach and Brunger<sup>44</sup> have recently solved the X-ray co-crystal structure of BoNT/A LC complexed with residues 141–204 of synaptosomal-associated protein 25 (SNAP25). This important structure reveals substrate-recognition exosites that could be exploited for inhibitor design. Toosendanin<sup>45</sup>, a triterpenoid natural product, might act at such an exosite.

The majority of compounds that inhibit BoNT/B metalloprotease activity are pseudo-peptidic in nature. However, two non-peptidic inhibitors have been described<sup>46,47</sup> (TABLE 6). With regard to pseudo-peptides, phosphoramidon and three of its synthetic derivatives were found to be weak inhibitors<sup>48</sup>, whereas buforin I has also shown activity against the BoNT/B LC<sup>49</sup>. Recently, a Cys-containing peptide inhibitor was also reported<sup>50</sup>. The most effective pseudo-peptide BoNT/B LC inhibitors to date were identified during the course of several complementary studies. Initially, a series of pseudo-tripeptides with nominal  $K_i$  values were generated<sup>51</sup>. In subsequent publications<sup>52,53</sup>, side-chain modifications produced more potent inhibitors (TABLE 6)<sup>53</sup>. In the latest study, the pseudo-tripeptide inhibitors were subjected to minor structural changes, and several compounds with  $K_i$  values ranging from 2.3 nM to 5.4 nM were generated, with a symmetrical disulphide derivative displaying the greatest potency (TABLE 6)<sup>54</sup>.

With regard to the BoNT serotype F LC, Schmidt and Stafford recently generated a potent peptidic inhibitor composed of VAMP residues 22–58 (J. J. Schmidt and R. G. Stafford, personal communication).

**Ricin toxin.** The potency of RICIN TOXIN is shown in TABLE 2. In preparation for inhibitor development, Monzingo and Robertus<sup>55</sup> solved co-crystal structures of two substrate analogues — formycin monophosphate (FMP) and dinucleotide ApG — bound to the ricin toxin A chain (RTA). Using the FMP–RTA co-crystal as a guide, Yan *et al.*<sup>56</sup> identified the pterin-based inhibitors pteric acid and neopterin (TABLE 7). Both inhibitors were co-crystallized with RTA (FIG. 2c). In a follow-up study<sup>57</sup>, an oxazole-pyrimidine ring system (9OG) (TABLE 7) was also found to inhibit the RTA. Aptamers (nucleic-acid ligand<sup>58</sup>) that inhibit the RTA have also been generated. Hesselberth *et al.*<sup>59</sup> identified a 31-nucleotide aptamer, whereas Tanaka *et al.*<sup>60</sup>, using a mechanistic approach<sup>61</sup>, generated a variety of much smaller aptamers containing unnatural sugar and purine derivatives (TABLE 7)<sup>60</sup>.

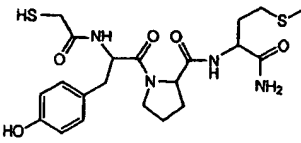
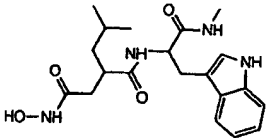
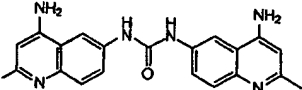
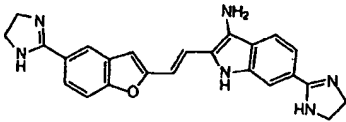
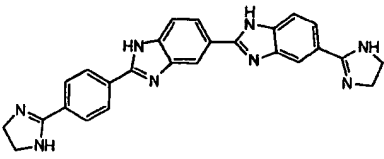
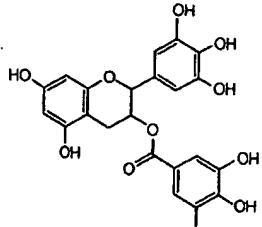
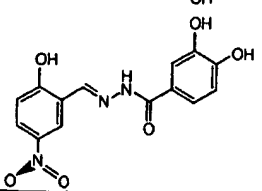
**Staphylococcal enterotoxins.** STAPHYLOCOCCAL ENTEROTOXINS (SEs) stimulate a powerful cytokine and immune response, which has earned them the name superantigens (SAGs). FIGURE 2d shows the co-crystal of a SAG and a human class II major histocompatibility complex (MHC) molecule. SEs and other related exotoxins have been implicated in various disorders and

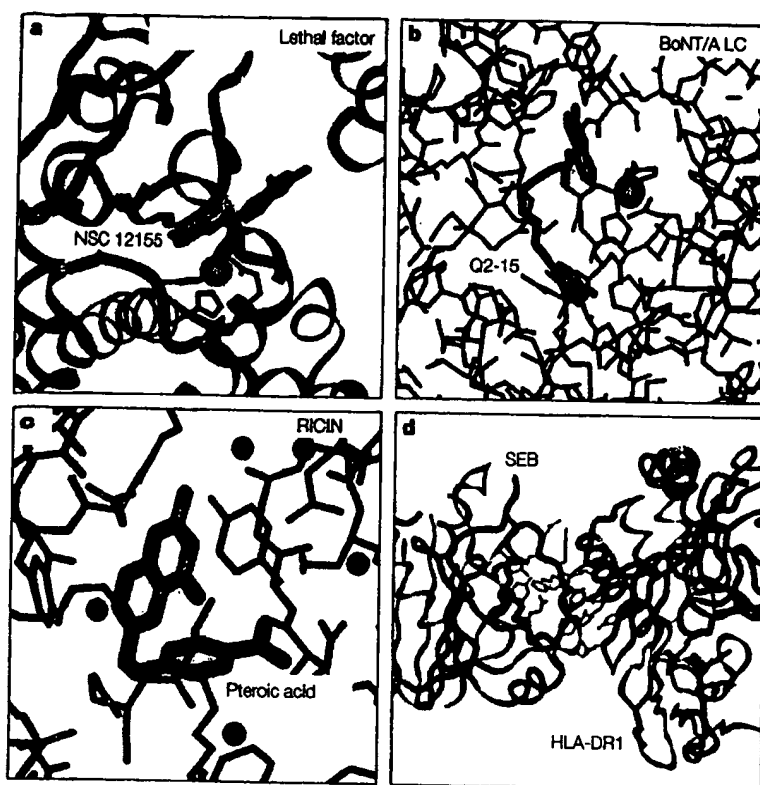
lethal shock syndrome<sup>62</sup>. Many of these exotoxins are relatively easy to produce in large quantities and are remarkably stable. When delivered by aerosol, these agents are highly incapacitating and lethal. Modulating cytokine responses is one of the clear mechanisms to interfere with SE toxicities<sup>63,64</sup>.

Soluble decoy receptor, high-affinity variants of the T-cell receptor (TCR) V $\beta$  region have been engineered to counteract SEs as therapeutic leads<sup>65,66</sup>. Additional studies have now generated V $\beta$  proteins against several toxins with picomolar affinities (R. Buonpane and D. Kranz, personal communication). Such high affinity might be essential for neutralizing agents such as SAGs, which are highly toxic even at extremely low concentrations.

Although consensus peptides as therapeutics are presently controversial, in some animal models these mimetic peptides have been shown to diminish the toxicity of SAGs<sup>67–69</sup>. In one such study, Arad and colleagues<sup>70</sup> used a mimetic peptide and produced evidence that divergent SAGs inhibited gene expression of human T<sub>H</sub>1 cytokines. In low molar excess over SAG challenge concentration, this peptide mimetic protected mice from the lethal effects of a broad spectrum of these toxins, even when given post-challenge. The peptide is a mimetic of a domain that is structurally conserved among SAGs, yet it is remote from binding sites for MHC class II and TCR. It has been proposed that SAGs might use this domain to bind to a novel receptor that is crucial for their action (Kaempfer R, personal communication).

Table 3 | Lethal factor (LF) inhibitors

LF inhibitor chemical structure	Name	<i>In vitro</i> activity	Cell-based assay	References
	2-thiolacetyl-YPM-amide	$K_i = 11 \mu\text{M}$		17
	GM6001	$K_i = 2.1 \mu\text{M}$	100 $\mu\text{M}$ concentration protects cells.	17
	NSC 12155	$K_i = 500 \text{ nM}$		19
	NSC 357756	$K_i = 4.9 \mu\text{M}$	100 $\mu\text{M}$ concentration protects cells.	19
	NSC 369721	$K_i = 4.2 \mu\text{M}$		19
	Epigallocatechin-3-Gallate	$\text{EC}_{50} = 97 \text{ nM}$	10 $\mu\text{M}$ protects cells.	21
	DS-998	$K_i = 1.1 \mu\text{M}$	1–10 $\mu\text{M}$ concentration protects cells.	20



**Figure 2 | Toxin interactions with inhibitors (a–c) or other proteins (d).** **a** | The co-crystal structure of NSC 12155 bound in the lethal factor (LF) substrate-binding cleft (PDB Ref Code = 1PWP). LF is shown in green ribbon. Residues of the LF catalytic engine are shown in stick. Carbon atoms are green; oxygen atoms are red; and nitrogen atoms are blue. NSC 12155 carbons are magenta. The inhibitor sits in close proximity to the enzyme's catalytic zinc (gold). **b** | Inhibitor Q2-15 docked in the botulinum neurotoxin serotype A (BoNT/A) light-chain (LC) substrate-binding cleft. The BoNT/A LC model is a dynamics conformation<sup>205</sup> generated from the X-ray crystal structure of PDB ref code = 1E1H. Colours are as described for **a**. Additionally, enzyme residues are rendered in stick. Q2-15 carbons are magenta; and Q2-15 chloro substituents are light green. One of the 7-chloro-quinoline components interacts with the catalytic zinc of the enzyme, whereas the other binds in a pocket located behind the catalytic engine of the enzyme. **c** | The co-crystal structure of pteric acid bound in the substrate-binding pocket of the ricin A chain (PDB Ref Code = 1BR6). Colours are as described for **a** and **b**. Red spheres are water molecules. **d** | The co-crystal structure of the SEB-HLA-DR1 interaction (PDB Ref Code = 1SEB). SEB is depicted as cyan ribbons and HLA-DR1 is depicted as green ribbons. The side chains of residues spanning the contact interface are shown in stick, with carbon colours corresponding to protein ribbon colour. Residue oxygens are red and nitrogens are blue.

### Targeting viral pathogens: variola and filoviruses

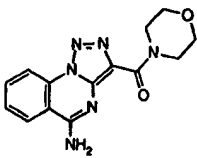
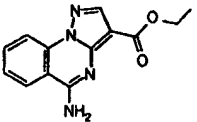
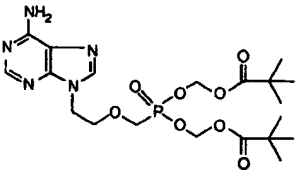
Therapeutics for viral infections can be broadly categorized as agents that attack the virus and its replicative cycle directly, or as agents that assist and fortify host immune defences. In principle, there are abundant targets and numerous strategies for both categories. TABLE 8 provides an overview of the strategies and opportunities available for new therapies against a virus, juxtaposed with some of the challenges in bringing such strategies into clinical use. The view presented is necessarily incomplete, but serves to highlight both the apparent vulnerabilities of viruses and the extraordinary challenges inherent to dampening logarithmic viral replication to a medically significant degree. As reviewed recently by De Clercq<sup>71</sup>, there are only 37 licensed antiviral drugs (not including interferons or

antibodies) available for clinical use. Many are for the treatment of HIV, 12 are for treating herpes virus (herpes simplex virus (HSV), Varicella-Zoster virus (VZV) and cytomegalovirus (CMV)) and 4 are for the therapy and prophylaxis of influenza virus. However, a cause for optimism is that the viruses of greatest concern in biowarfare and bioterrorism cause acute viral infections, which for lucky survivors is followed by immune recovery. Antiviral therapies therefore need only be effective for relatively short periods (see BOX 1 for case examples of filoviruses and orthopoxviruses).

**Antiviral drugs.** Attachment and entry remain enigmas for both filoviruses and orthopoxviruses, and emerging data are mired in uncertainty and controversy. The search for specific filovirus receptors<sup>72,73</sup> has been countered by evidence of more ubiquitous and unspecific lectin-like receptors<sup>74,75</sup> that might be difficult to antagonize with drugs. However, recent structure-activity relationship (SAR) studies indicate that Cyanovirin-N, a carbohydrate-binding protein, might inhibit Ebola virus entry<sup>76</sup>. Orthopoxviruses, though very different in their surfaces from the sugary filaments of Ebola and Marburg, are similarly the subject of viral attachment and entry research<sup>77</sup>. Fusion inhibition, which has proven fruitful for treating both HIV and influenza<sup>71</sup>, could provide therapeutic opportunities for both viral genera, and is being actively pursued<sup>77–80</sup>. Inhibition of viral replication seems to be especially feasible for both filoviruses and orthopoxviruses: numerous genomes have been sequenced, several key enzymes identified, basic replicative steps described and structural associations among proteins partially described<sup>77,81</sup>.

This abundance of potential targets could result in several therapeutic approaches, including antisense targeting of the viral genome, inhibition of the replicase or polymerase activity by small-molecule inhibitors, as well as other specific molecular targets essential for the formation of a replication-competent complex<sup>82</sup>. The recent development of reverse genetics and filovirus reporter-based mini-genomes<sup>83</sup>, as well as green fluorescent protein (GFP)-expressing Ebola virus<sup>84</sup>, is expected to significantly facilitate the identification of inhibitors of filovirus replication. Final assembly and viral egress from cells is simpler for filoviruses than for poxviruses. Results from electron microscopy have long indicated that the final assembly of filamentous Ebola and Marburg viruses occurs at cell membranes<sup>85,86</sup>, and recent work has shown that filoviruses are among the subset of viruses that exploit specialized cell-membrane regions called lipid rafts<sup>87</sup>. Filovirus raft assembly might therefore be a viable target. Reverse genetics experiments can be used to explore whether a putative target, such as furin cleavage site of Ebola virus, is essential for viral infection<sup>88</sup>. Compared with filoviruses, poxvirus egress from cells is considerably more complicated<sup>77</sup>, a situation that would seem to make the target even more vulnerable. Over the years, vaccinia virus mutants defective in various aspects of final assembly have been identified, host proteins implicated and

Table 4 | Oedema factor (EF) inhibitors

EF inhibitor chemical structure	Name	<i>In vitro</i> activity	Cell-based assay	References
	119804	IC <sub>50</sub> = 60 μM	125 μM and greater prevents cAMP-induced cell rounding.	23
	277890	IC <sub>50</sub> = 90 μM	125 μM and greater prevents cAMP-induced cell rounding.	23
	Adefovir dipivoxil	K <sub>i</sub> = 27 nM (for adefovir diphosphate, active cellular metabolite of adefovir dipivoxil)	IC <sub>50</sub> = 0.1–0.5 μM	24

compounds identified that inhibit late particle formation. Additionally, the apparently effective but problematic antiviral drug cidofovir seems to be effective against many orthopoxviruses, and is potentially useful for the treatment of smallpox and vaccinia<sup>71,89</sup>.

**Adjunctive therapy.** Filovirus infections are associated with a number of pathological conditions, including disseminated intravascular coagulation, which has been proposed to result from upregulation of tissue factor on the surface of leukocytes<sup>90</sup>. Partial success against Ebola virus infections in rhesus monkeys using recombinant nematode anticoagulant protein C2 has recently been reported<sup>91</sup>. Although this study is encouraging, the utility of anticoagulant therapy in humans requires further studies — in particular in combination with specific antiviral therapeutics.

**Therapeutic antibodies.** Both filoviruses and orthopoxviruses illustrate how the potential complexity and effectiveness of antibody-mediated protection is so often underestimated. Viral neutralization — commonly interpreted to mean the capacity of an immunoglobulin to interfere with viral attachment or entry — is only part of the protective role of antibodies<sup>92</sup>, and is sometimes insufficient.

In rodent models of lethal Ebola and Marburg viruses, the administration of both polyclonal and monoclonal antibodies unambiguously confers protection before and sometimes after viral infection, and the demonstration of virus-neutralizing activity in the transferred antibody is a poor predictor of its efficacy *in vivo*<sup>93–96</sup>. The few antibodies tested in sensitive non-human primate models of filovirus infection have delayed viraemia and death, but have not been fully preventative when the viral challenge was robust<sup>97</sup>. This has led to premature assertions about the irrelevancy of antibodies as filovirus therapies. Lessons from viral vaccine studies with Ebola and Marburg viruses repeatedly show

that antibodies to the viral glycoprotein in conjunction with T-cell responses to this and other proteins are required for optimal protection<sup>94,98–102</sup>. Attempts to influence clinical outcomes in humans by the transfer of plasma from convalescent to ill individuals produced encouraging results<sup>103,104</sup>, but these studies were inadequately controlled and therefore inconclusive.

A common observation in orthopoxviruses is the production of neutralizing antibodies (raised against inactivated virus) that alone prove insufficient to prevent disease and death, but which are protective when combined with an additional antibody population (found in serum from animals that had been infected with live virus)<sup>105</sup>. We repeated this observation both with monoclonal antibodies<sup>106</sup> and with DNA vaccines that evoked antibodies<sup>106,107</sup>; in this case, even the most potent neutralizing antibodies (against the vaccinia virus protein L1R) were insufficient to prevent the inexorable spread of virus in infected animals. In contrast, an antibody to a virally encoded cell-surface protein (A33R) was sufficient by itself or in conjunction with anti-L1R to provide robust protection from vaccinia virus in rodents. Others, extending the observations to additional proteins, have reported similar findings<sup>108</sup>, and an experimental DNA vaccine against monkeypox virus in non-human primates yielded concordant results<sup>107</sup>. This raises a question: how might antibodies, in addition to neutralizing antibodies, confer a therapeutic effect? Early observations<sup>92,109</sup> implicated the capacity of antibodies to bind to viral proteins on the surface of infected cells, and subsequent observations, including those with filoviruses and orthopoxviruses, tend to be consistent with the proposed requirement that the targets of non-neutralizing antibodies be externally exposed. Mechanistically, one might evoke complement-mediated lysis of cells, antibody-dependent cellular cytotoxicity (in which Fc receptor-bearing cells destroy virally infected cells), perturbation of late events in viral assembly (as in the drug targeting above) or, as

in the case of orthopoxviruses, the targeting of a particularly important but quantitatively minor viral population<sup>105,108</sup>. In terms of the therapeutic value of antibodies, complexity is added by the search for antibodies in addition to those that can be assayed rapidly by binding or neutralization. Historically, the potency of vaccinia immune globulin (licensed for the treatment of smallpox vaccine complications) was judged by its neutralization capacity, a strategy salvaged by the acquisition of antibodies from donors whose sera also contained many other antibodies as well<sup>110</sup>.

**Augmenting or protecting innate immunity.** The goal of some antiviral agents is to tip the balance of the immune response towards innate immunity and allow specific immune clearance mechanisms (adaptive immunity) to take over<sup>111</sup>. At the crossroads of many innate immune responses are interferons, a family of molecules that can directly evoke antiviral responses. However, the utility of interferons as broad-spectrum antivirals has been limited both by the transience and the toxicity of their effects. This has engendered caution about the prospects for a broad array of other newly described cytokines that also stimulate innate immunity. On the other hand, other opportunities for drug intervention have arisen in targeting viral pathogens. The identification of proteins produced by vaccinia and influenza virus that act as interferon antagonists<sup>112,113</sup> was followed by the demonstration that Ebola<sup>114</sup> and Marburg<sup>115</sup> viruses also make interferon antagonists. Additionally, orthopoxviruses synthesize an impressive array of homologues of cytokines, cytokine receptors, complement proteins, growth hormones and other molecules — the effects of which could confound innate immune responses<sup>116</sup>. Our ability to modify the innate immune response in a therapeutically significant manner necessitates a deeper understanding of the role of the components of this arm of the immune system in specific viral infections.

Recently, a crucial role for natural killer (NK) cells was defined in protection against Ebola infection<sup>117</sup>. Interestingly, adoptive transfer of NK cells treated with Ebola virus-like particles and not inactivated Ebola virus resulted in significant protection of mice against lethal challenge, indicating that mobilizing the effector innate response early in infection might be a promising therapeutic strategy against filoviruses.

**Targeting host pathways.** Viral pathogens have evolved over millennia by adapting to a limited number of cellular mechanisms for cellular entry, replication, assembly and budding. Although a tremendous amount of effort has been devoted in the past decades to the development of therapeutic strategies targeting virus components, half of this work involves a single virus (HIV). In contrast, the common cellular pathways used by a wide array of viruses have been largely neglected as therapeutic targets. In this regard, genetically engineered microbes represent major challenges for biodefence both because the pathogenicity of the organism might be unrecognized and/or the pathogenicity might be tailored to counter existing pathogen-targeted therapeutics. Host-targeted therapeutics would be the most viable option in coping with such unpredictable challenges. Such host-targeted therapeutics would have two advantages: they would act as broad-spectrum therapeutics and block all of the viruses that use the affected pathway; and they would make it more difficult for the pathogen to develop resistance, because there would be few alternative cellular pathways available for the virus to take advantage of. Besides cellular receptors and co-factors, a number of intracellular pathways, such as the vacuolar protein-sorting machinery<sup>118</sup>, cytoskeletal network<sup>119</sup> and components of cellular antiviral defence<sup>120,121</sup>, have been identified as crucial for viral pathogenesis. However, despite these advances, our understanding of the host pathways involved in viral pathogenesis remains limited. Genetic approaches such

Table 5 | **Botulinum serotype A light chain (BoNT/A LC) inhibitors**

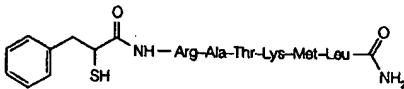
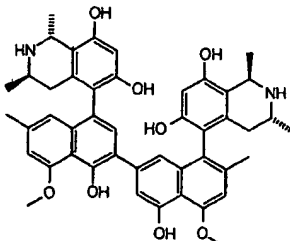
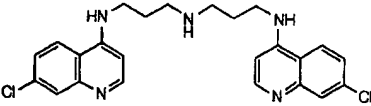
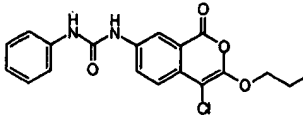
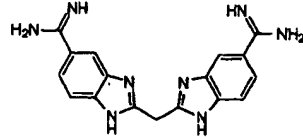
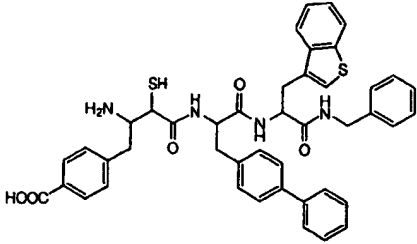
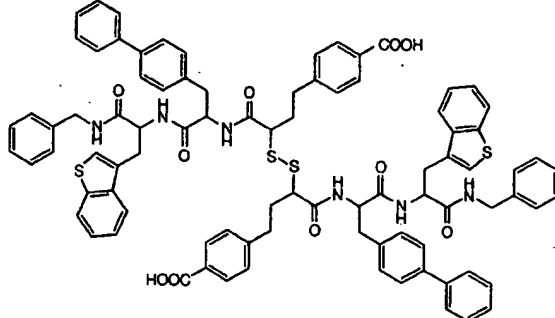
BoNT/A LC inhibitor chemical structure	Name	In vitro activity	References
	2-mercapto-3-phenylpropionyl-RATKML-amide	$K_i = 330 \text{ nM}$	36
	Michellamine B	62% inhibition, 20 $\mu\text{M}$ concentration.	41
	Q2-15	60% inhibition, 20 $\mu\text{M}$ concentration.	41

Table 6 | **Botulinum serotype B light chain (BoNT/B LC) inhibitors**

BoNT/B LC inhibitor chemical structure	Name	In vitro activity	References
	ICD-1578	IC <sub>50</sub> = 27 μM	46
	BABIM	IC <sub>50</sub> = 5–10 μM	47
	Biphenyl/benzothienylmethyl derivative	K <sub>i</sub> = 20 nM	52
	Bis-derivative	K <sub>i</sub> = 2.3 nM	54

as RNA interference (RNAi), as well as various physical and functional knockout technologies, need to be applied to identify host genetic pathways involved in viral pathogenesis and to establish the degree of commonality of these pathways across viral families. Molecular details of these pathways and the nature of their interactions with viral components need to be intensively studied by genetic, biochemical, structural and modelling approaches. This detailed body of knowledge would serve as a basis for identifying host targets and the rational design of broad-spectrum therapeutic strategies.

#### Existing antimicrobial treatments

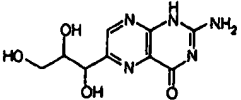
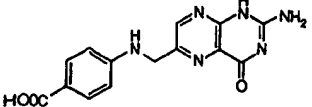
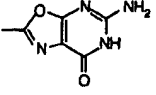
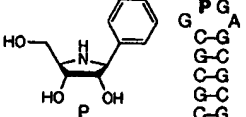
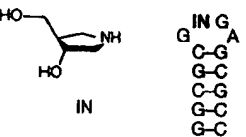
At this time there are therapeutic protocols for treating those infected with many of the bacterial biowarfare pathogens. However, the scope of recovery is variable — in the case of individuals infected with inhalational anthrax, there is a limited window of opportunity during which antibiotics will control and eliminate the infection. This section of the review covers characteristics (TABLE 9) and current drug therapies for three biowarfare agents: anthrax, plague and tularaemia.

Naturally occurring strains of *B. anthracis* are generally susceptible to penicillins, first-generation

cephalosporins, tetracyclines, rifampin, aminoglycosides, vancomycin, clindamycin and fluoroquinolones. It was recently found that 20 strains of *B. anthracis* also show sensitivity to imipenem, meropenem, daptomycin, quinupristin-dalfopristin, linezolid, GAR936, BMS284756, ABT773, LY333328 and resistance to clofazamine<sup>122,123</sup>. The CDC and the Working Group for Civilian Biodefense treatment guidelines have been published for treatment of pulmonary anthrax<sup>124</sup>, and are provided in TABLE 10. The choice of the second or third antibiotic should be influenced by the likely resistance pattern of the strain causing the infection, and consideration should be given to antibiotics that penetrate the blood–brain barrier (penicillins and carbapenems, for example) due to the high frequency of meningitis associated with inhalational anthrax exposure<sup>125</sup>. The duration of therapy is controversial, but involves at least 60 days of treatment<sup>124,125</sup>. Corticosteroids have been mentioned as a possible adjunctive therapy in the setting of meningitis or severe mediastinal oedema<sup>125</sup>, but there are no data to definitively support their use.

A major concern with regard to *B. anthracis* and other microbial biodefence agents is genetically engineered antibiotic resistance. Several reports of recombinant

Table 7 | Ricin toxin chain A (RTA) inhibitors

RTA inhibitor chemical structure	Name	In vitro activity	References
	Neopterin	$K_i = >2 \text{ mM}$	56
	Pteric acid	$K_i = 0.6 \text{ mM}$	56
	9OG	$IC_{50} = 0.4 \text{ mM}$	57
	P-14	$K_i = 0.18 \text{ }\mu\text{M}$	60
	1N-14	$K_i = 0.48 \text{ }\mu\text{M}$	60

**YERSINIA PESTIS**

The causative agent of plague, it is an aerobic, Gram-negative bacillus from the bacterial family Enterobacteriaceae.

plasmids that confer antibiotic resistance when inserted into *B. anthracis* have been published. One plasmid-containing strain was resistant to tetracycline, doxycycline and minocycline<sup>126</sup>. In another study, a recombinant plasmid encoding for resistance to penicillin, tetracycline, chloramphenicol, rifampin, macrolides and lincomycin was inserted into the *B. anthracis* strain STI-1, which reportedly stably inherited the plasmid over several generations<sup>127</sup>. The possibility of antibiotic resistance in this pathogen indicates the

importance of initial combination therapy when exposure to a genetically modified strain is suspected.

*YERSINIA PESTIS* is typically susceptible *in vitro* to penicillins, many cephalosporins, imipenem, meropenem, aminoglycosides, amikacin, quinolones and tetracyclines. It is variably susceptible to trimethoprim, chloramphenicol and rifampin, and is commonly resistant to macrolides, clindamycin, novobiocin, quinupristin-dalfopristin and clofazamine (H. Heine, personal communication). (See TABLE 10 for recommended antibiotic treatments for pneumonic plague.) The preferred therapy for *Y. pestis* infection is an aminoglycoside, with streptomycin as an FDA-approved medication and gentamicin often mentioned as an alternate antibiotic.

Although rarely reported, naturally occurring, highly antibiotic-resistant strains of *Y. pestis* do occur. In a recent report, a strain isolated from a boy in Madagascar was demonstrated to have acquired a plasmid that mediated resistance not only to streptomycin, chloramphenicol and tetracycline, but also to ampicillin, sulphonamides, kanamycin, spectinomycin and minocycline. These naturally occurring, highly resistant antibiotic strains are extremely concerning with respect to the development of biological weapons.

*FRANCESELLA TULARENSIS* is generally susceptible *in vitro* to aminoglycosides, tetracyclines, rifampin and chloramphenicol<sup>128-133</sup>; however, many strains seem to be resistant to  $\beta$ -lactam and monobactam antibiotics<sup>133</sup>. (See TABLE 10 for recommended tularemia treatments.) Similarly to the treatment of plague, streptomycin or gentamicin are the preferred therapy when there are no contraindications to the use of these medications<sup>134,135</sup>. Ciprofloxacin was effective in treating a recent tularemia outbreak in Spain<sup>136</sup>.

**Rapid detection and diagnostics**

The early detection and diagnosis of infection or intoxication with biological select agent and toxin (BSAT) is essential if intervention is to occur at a point at which the prognosis can still be influenced, and also to guide the selection of the optimum therapeutic protocol (TABLE 10). In addition, such information can greatly facilitate the logistics of mobilizing supplies and personnel to areas of exposure. Here, 'detection' is defined as including those technologies required to identify a biological threat in the environment before or coincident with exposure. Environmental detection usually involves the testing of air, soil, fomites, water and foodstuffs. 'Laboratory diagnosis' includes those methods used to confirm the clinical observations of a physician by evaluation of standard clinical specimens, such as blood, serum, exudates, saliva, stool and tissues (TABLE 9). The necessity for the rapid detection of BSAT-related illness and intervention with optimal therapeutic protocols was well illustrated during the 2001 anthrax attacks (BOX 2).

**Challenges facing the National Laboratory Response Network.** In 1999 a national laboratory response network (LRN) for bioterrorism was established by the CDC to test for biological and chemical agents (see FIG. 3

**Box 1 | Case examples of filoviruses and orthopoxviruses**

The filoviruses (Ebola and Marburg viruses) and the orthopoxviruses (variola/smallpox, monkeypox and other pox viruses) are high-priority viral threats, and there is an acute need for therapeutics that target these pathogens. Ebola and Marburg viruses are exceptionally deadly (70–90% mortality in some outbreaks), but are relatively simple viruses, consisting of seven genes encoded in a single strand of RNA<sup>159</sup>. They are moderately contagious, but otherwise have numerous characteristics commonly associated with biological weapons<sup>160</sup>. These viruses are endemic in Africa<sup>161,162</sup> and, despite a great deal of scientific progress in the past 10 years<sup>81,93,95,96,98,163–165</sup>, no vaccines or treatments are available for clinical use. For comparison, orthopoxviruses are large DNA viruses that have nearly 200 genes and some of the most complex viral replication cycles known. Variola virus, which causes smallpox, is the most feared of this genus<sup>166</sup> because it is highly contagious, incapacitating, disfiguring and potentially deadly (historical highs of around 40% mortality in unvaccinated persons). Monkeypox, a rodent virus endemic in Africa, is far less contagious than variola but in some outbreaks has caused up to 10% mortality in unvaccinated individuals. The classical smallpox vaccine, which consists of vaccinia virus, affords relatively robust protection against both variola and monkeypox viruses, but has proven problematic in the modern era not only because of previously known adverse reactions (including disseminated vaccinia), but because of a rediscovered association with myocarditis<sup>167,168</sup>. However, safer vaccines are in the research pipeline<sup>107,169</sup>. Vaccinia immune globulin, an antibody-containing product from vaccinated persons, was licensed and is now offered under investigational status for the treatment of disseminated vaccinia<sup>89</sup>.



Table 8 | **Quenching viral replication: opportunities and challenges**

Viral event	Dynamic consequences	Opportunities	Challenges
Virus (free or extracellular)	Gradual inactivation in a cell-free environment.	Specific binding and harmless removal of virus — for example by antibodies, heteropolymers and small molecules. Specific binding by drug or antibody to destabilize or irreversibly stabilize coat.	Phenotypic variation of viral population (quasi-species). Possibility of enhancing uptake and therefore disease. Natural diversity of coats among viral species and strains.
Attachment to cells	Binding of virus to receptor(s) or unspecific ligands. Earliest signalling of innate immunity.	Receptor blockade by antibodies or other drugs. Deliberate prior activation of innate immunity.	Genotypic and phenotypic variation present in an amplifying virus population and escape mutants. Redundancy and degeneracy in viral and cell receptors. Adverse and transient effects of activating innate immunity.
Entry, fusion, release of viral genome, translocation	Penetration and unveiling of RNA or DNA, sometimes stepwise or compartmentalized. Early defence cascades activated, including interferons, RNAi, apoptosis.	Binding to fusion domain, fusion inhibition. Targeting exposed RNA, DNA, for example, nuclease, antisense.	Fusion domains often cryptic, and are only transiently accessible. Nucleic acids protected by viral proteins, compartmentalization.
Complex cycle of transcription, translation and genome replication	Cell defence cascades are amplified; viral antagonists of intra- and extracellular defences produced; viral proteins on cell surfaces expressed; viral proteins secreted; MHC-associated viral peptides processed; perturb 'normal' cell surface.	Specifically bind/disrupt viral protein/nucleic-acid functions and interactions (for example, protease inhibitors and replicase inhibitors); competitively inhibit viral antagonists of innate and adaptive immunity; specifically target viral proteins on cell surfaces (for example, antibodies for ADCC or targeted toxin); exploit, amplify and influence the innate and adaptive responses (for example, NK cells and CTL) to eliminate 'modified self'.	Delivery of active compounds to intracellular targets; identification of appropriate targets; escape mutants, variation among viral strains; insufficient knowledge of how to safely manipulate the immune system without exacerbation of disease and autoimmunity in some individuals.
Pre-assembly: an orchestrated, compartmentalized encapsidation of nucleic acid with viral proteins	Specific or quasi-specific associations between viral proteins and nucleic acids.	Identify and inhibit protein-protein interactions; perturb nucleic-acid encapsidation motifs (for example, using a drug antagonist or antisense).	Delivery of active compounds to intracellular targets; identification of appropriate targets.
Final assembly: can involve translocation, acquisition of outer capsid and/or budding from cell membranes	Self-assembly driven by specific binding and movement of proteins; preferential assembly in specialized proteins (for example, lipid rafts); exploitation of cellular proteins and pathways (for example, TSG101); cell exhaustion and apoptosis; and death.	Bind and disrupt proteins involved in final packaging; reversibly perturb essential cellular sites and proteins.	Delivery of active compounds to intracellular targets; identification of appropriate targets; overall safety of compounds that disrupt cellular processes.
Repetition <i>in vivo</i> , manifested by tissue tropisms, damage and disease	Logarithmic amplification of viral burden; fatal virus-induced lesions in crucial organs; triggering of 'cytokine storms'; immunopathology from potent but lagging response.	Treat symptoms to sustain victim until immune system prevails; manage immune response and cytokine polarity.	Discovery of active compounds; addressing issues of drug or antibody pharmacokinetics, bioavailability, efficacy, feasibility and safety.

for a schematic of the process) that could be used during a terrorism incident<sup>137,138</sup>. Each laboratory in the LRN follows the same rules for sample collection, shipping, agent containment and testing. LRN laboratories maintain secure communication channels among themselves, state and local health authorities, CDC and other federal agencies. The mission of the LRN is to maintain a laboratory network that will quickly respond to acts of biological and chemical terrorism. The system is now organized into a collection of surveillance (previously known as level A), confirmatory (level B and C) and national laboratories (level D).

FDA-approved assays do not exist for most BSAT. The CDC therefore provides LRN-registered clinical laboratories, which are the front-line laboratory responders to biological terrorism, with approved protocols for most of the category A agents and some category B agents. LRN protocols use an integrated

system of well-established microbiological methods, PCR gene amplification and improved immunodiagnostic assays<sup>139</sup>. CDC-supplied reagents and standards exist for the identification of *B. anthracis*, BoNT/A, *Y. pestis*, *F. tularensis* and *Brucella* spp. For a large number of agents, specimens must be sent directly to the CDC in Atlanta, Georgia, USA, or to designated LRN reference laboratories because of the extreme hazard they represent to clinical laboratory personnel and the technical complexity of the analysis required. In most cases the LRN system requires a combination of a screening evaluation at the level of the local hospital clinical laboratory and confirmation by a hierarchical reference laboratory in the system. TABLE 10 shows the estimated time required for conducting LRN protocols, assuming a low-complexity sample or specimen. We can expect that the time required for laboratory confirmation will be worse for samples that must be transported to the

**FRANCESSELLA TULARENSIS**  
The causative agent of tularaemia, it is a small, aerobic Gram-negative coccobacilli. This agent is the most infectious human pathogen known. In the past, both the former Soviet Union and the US had programmes to develop weapons containing this bacterium.

Table 9 | Characteristics of selected BSAT

BSAT	Biological characteristics	Clinical specimens	Diagnostic methods*
Anthrax	Gram-positive rod; spore-forming; aerobic; non-motile; catalase positive; large, gray-white to white, non-haemolytic colonies on sheep-blood agar plates.	Blood; cerebral spinal fluid; pleural effusion fluid; skin-lesion material such as vesicular fluid or eschar.	Culture; $\gamma$ -phage sensitivity; immunohistochemistry; PCR.
Botulism	Gram-positive rod; spore-forming; obligate Anaerobe; catalase negative; lipase production on egg yolk agar; 150-kDa protein toxin (types A–G); 2 subunits.	Serum; gastric aspirates; stool; respiratory secretions.	Culture; immunoassay; mouse neutralization assay; PCR.
Plague	Gram-negative coccobacilli often pleomorphic; non-spore forming; facultative anaerobe; non-motile; beaten copper colonies (MacConkey's agar).	Lymph node smears; aspirates; sputum; blood; cerebral spinal fluid.	Culture; immunofluorescence assay; PCR.
Smallpox	Large double-stranded DNA virus; enveloped, brick-shaped morphology; Guarnieri bodies (virus inclusions) under light microscopy.	Throat swabs; induced respiratory secretions; serum; aspirates; tissue scrapings.	Viral culture; PCR; EM; immunohistochemistry; immunoassay.
Tularaemia	Extremely small, pleomorphic, Gram-negative Coccobacilli; non-spore forming; facultative intracellular parasite; non-motile; catalase positive; opalescent smooth colonies on cysteine heart agar.	Blood culture; serum; ulcer material; conjunctival exudates; sputum; gastric washes; pharyngeal exudates.	Culture; PCR; immunoassay.
Ebola and Marburg	Linear, negative-sense single-stranded RNA virus; enveloped; filamentous or pleomorphic, with extensive branching, or U-shaped, 6-shaped or circular forms; limited cytopathic effect in Vero cells.	Serum; liver; spleen; lymph nodes; kidney; lung; and gonads.	Viral culture; PCR; EM; immunoassay; immunohistochemistry.
Viral encephalitis	Linear positive-sense single stranded RNA virus; enveloped, spherical virions with distinct glycoprotein spikes; cytopathic effect in Vero cells.	Throat swabs; serum; cerebrospinal fluid.	Viral culture; PCR; EM; immunoassay; immunohistochemistry.
Ricin toxin	60–65 kDa-protein toxin; two subunits; castor bean origin.	Serum; stool; urine; spleen, lung, kidney.	Immunoassay.

\*Includes screening methods and confirmatory assays supplementing standardized protocols in the US National Laboratory Response Network. BSAT, Biological Select Agent and Toxin.

centrally responding CDC laboratory after screening at the local level, as required for smallpox and haemorrhagic fevers. On the basis of the limited public reports of the 2001 response to anthrax attacks, the calculated median time from first medical visit to laboratory confirmation for suspected cutaneous and inhalation anthrax cases ( $n = 22$ ) was 9 days<sup>140,141</sup>. In most of the cases, in which an optimal antibiotic set was initiated as the first therapeutic option, the diagnosis depended on the astute observations and the sensitivity of the attending physician to the possibility of anthrax. Although the laboratory response has technically improved since 2001, the reaction to an unknown or a genetically engineered threat could mimic the 2001 experience.

**Watching and sensing for biothreat agents.** Two federally sponsored programs, BioWatch and BioSense, are in the early stages of implementation and will encourage the recognition of biological threat attacks on a wide scale<sup>142,143</sup>. The BioWatch Program, which is a collaborative program between the Environmental Protection Agency (EPA), the Department of Homeland Security (DHS), the CDC and local authorities, will provide round-the-clock environmental monitoring for the intentional airborne release of select biological threats. Solid-phase filters and sometimes aqueous concentrates from BioWatch air samplers are evaluated for the presence of pathogens by designated local or

state public-health laboratories using LRN protocols and assays. Similar surveillance systems are planned for post offices, and research has begun to devise systems to protect buildings using 'smart' monitoring systems<sup>144,145</sup>. Presumably after confirmation of the intentional release of a biological agent, local officials will implement a response plan that might include widespread prophylaxis and treatment in accordance with the public-health threat. The BioSense Program will use epidemiological methods to monitor selected surrogate markers of infectious disease outbreaks, such as emergency room visits, absentee rates at schools and work, pharmacy visits and other indicators. Possible limitations for both BioWatch and Biosense are described in BOX 3.

**Traditional immunodetection.** The detection of agent-specific antibodies has been a traditional method to confirm clinical diagnoses. Others have demonstrated assays for the rapid detection of anthrax-specific antibodies in patient sera<sup>146</sup>. Recently, the FDA approved the use of the first commercial assay that detected anthrax-specific antibodies with high sensitivity and specificity. Although these assays are sensitive for detecting anthrax-specific antibodies in highly immunized individuals and convalescent sera, they might not be effective for identifying patients in the early stages of disease. Among postal workers, who arguably received

Table 10 | Requirements for rapid diagnosis

BSAT	CDC category	Incubation period <sup>a</sup>	Disease duration <sup>a</sup>	Diagnostic approaches <sup>a</sup>	Time to diagnosis <sup>a</sup>	Therapeutic options
Anthrax	A	1–6 days	Death in 3–5 days (untreated)	Level A Protocol	18–24 h	Ciprofloxacin, doxycycline, penicillin
Botulism	A	1–5 days	Death in 24–72 h (untreated); 30–60 days w/treatment	Level A Protocol	3–21 days	Equine & human antitoxin
Plague	A	2–3 days	1–6 days (usually fatal)	Level A Protocol	2 days	Tetracycline, doxycycline
Smallpox	A	7–17 days	4 weeks	Level D Protocol	24–48 h	Vaccinia vaccine, cidofovir
Tularaemia	A	1–21 days	>2 weeks	Level A Protocol	3 days	Streptomycin, gentamicin
Ebola	A	4–21 days	7–16 days (usually fatal)	Level D Protocol	1–3 days	Supportive care
Marburg	A	9–10 days	5–14 days (usually fatal)	Level D Protocol	1–3 days	Supportive care
Brucellosis	B	5–60 days	>8 weeks to >1 year	Level A Protocol	14–21 days	Doxycycline and rifampin
Glanders	B	10–14 days	7–10 days	Classical Protocol	1–3 days	Sulphadiazine, tetracyclines, ciprofloxacin, streptomycin, novobiocin, gentamicin, imipenem, ceftazidime
Q Fever	B	10–40 days	2–14 days	Classical Protocol	7–14 days	Tetracycline, doxycycline
Viral encephalitides	B	2–6 days	2–21 days	Level D Protocol	1–3 days	Supportive care
Ricin toxin	B	18–24 hr	1–12 days	Level D Protocol	1–5 days	Supportive care

<sup>a</sup>Adapted from REF 137. <sup>b</sup>From the Emergency Preparedness and Response website of the Centers for Disease Control and Prevention (<http://www.bt.cdc.gov>). Surveillance laboratories (level A); national laboratories (level D)

the highest dose of anthrax spores during the 2001 anthrax attacks, the mean duration between exposure and onset of disease was 4.5 days. Disease onset in these cases would be prior to the development of a robust humoral antibody response. Moreover, the need to collect paired acute and convalescent sera could limit the usefulness of these assays as epidemiological tools.

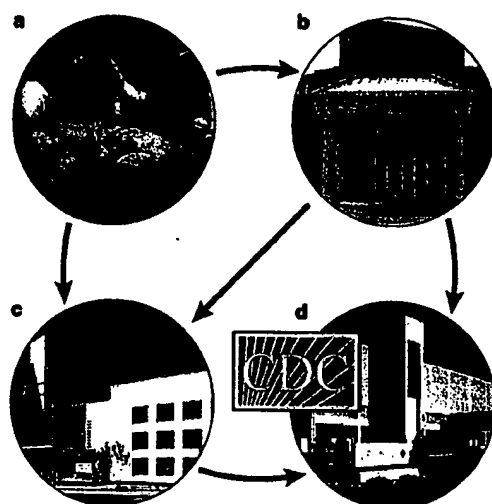
**Bioagent-directed detection.** Promising new technologies could enable the early recognition of replicating aetiological agents and their virulence factors. Potentially, the amplification of variable gene regions flanked by conserved sequences, followed by electrospray ionization mass spectrometry and base-composition

analysis of the products could be one approach. This approach, called triangulation identification for genetic evaluation of risks (TIGER)<sup>147</sup>, provides a high-throughput, multiple detection and identification system for nearly all known, newly emergent and bioengineered agents in a single test. This rapid, robust and culture-free system has been used to identify agents such as severe acute respiratory syndrome (SARS)-related coronavirus before their recognition by traditional methods. Robust and portable systems have been proposed for the development of civilian and military applications.

Biosensing represents another evolving mechanism for early detection. Here, single proteinaceous nanometer-scale pores (such as anthrax PA) can be easily applied to provide the physical basis for rapid biosensing applications. The mechanism of nanopore-based detection is simple: analytes that either bind to the nanopore or thread through it alter the ionic current in a characteristic manner. For example, the reversible binding of hydronium and deuterium ions to the  $\alpha$ -hemolysin ion channel causes current fluctuations with amplitude and spectral signatures that indicate the type and concentration of the isotope that is present<sup>148</sup>. The same ion channel was also used to detect and characterize individual molecules of single-stranded DNA that are driven electrophoretically through the pore<sup>149</sup>. This latter technology was used to detect other analytes in solution.

#### Box 2 | The necessity for BSAT rapid detection

During the 2001 anthrax attacks, although several patients had exposure to anthrax spores confirmed by nasal swab culture, no cases of disease occurred in the ~32,000 Senate staff and postal workers immediately targeted for post-exposure chemoprophylaxis<sup>146,170</sup>. By contrast, the post-syndromic group of inhalation anthrax-infected patients had a case mortality rate of approximately 45%<sup>141</sup>. These data were consistent with other studies that suggested that early and aggressive treatment is necessary to influence survival after exposure to inhalation anthrax<sup>171,172</sup>.



**Figure 3 | A schematic of how components of the national laboratory response network (LRN) coordinate when detecting and diagnosing a bioterror agent.** a | Initial responders collect evidence, which is then sent to surveillance laboratories or to confirmatory laboratories directly (b,c). Cooperation between these laboratories facilitates first line response procedures. d | Further confirmation of agent type and area of distribution, is then conducted at national laboratories.

Specifically, analytes of interest that bind to sites on pore-permeant polynucleotides alter the ability of the DNA to enter and thread through the pore<sup>150</sup>. These approaches can be extended into biosensing of anthrax toxin at pM amounts (J. Kasianowicz and K. Halverson, personal communication).

### Box 3 | BioWatch and Biosense limitations

Although the BioWatch and BioSense Programs represent significant improvements in biological defence readiness, they could, however, fail to influence morbidity and mortality in the case of an attack. BioWatch will probably document an attack when a biological threat agent is used on the scale of a weapon of mass destruction. However, extensive epidemiological surveillance might still be necessary before wide-scale prophylaxis is implemented. Another issue that is yet to be resolved is whether environmental sampling is sufficient to trigger a wide-scale medical response. The FDA might have to review environmental detection technologies if they influence medical decision-making. A measured and conservative approach is likely. During the 2001 anthrax attacks, Senate workers were screened and successfully treated after initial environmental test results were confirmed. But treatment of other populations might have been delayed by confusion and the lack of reliable laboratory confirmation<sup>146</sup>. BioWatch samplers might not be effective for limited attacks on individuals or contamination of water and food sources. In the case of BioSense, surrogate markers of infectious disease outbreaks can only be lagging indicators of an attack. There could be hundreds or thousands of cases before an outbreak is recognized, depending on the sensitivity of the final system. Smallpox virus has a comparatively long incubation time of up to 17 days (TABLE 10). By the time BioSense detects a smallpox attack, multiple foci of infection across the country, with coincident close contact spread, would most likely already be developing using current disease models<sup>173,174</sup>.

**Host-directed detection.** A powerful approach for identifying exposed or infected individuals is to develop highly specific and extremely sensitive innate biomarkers that can be detected very early after exposure to a biological agent. There are a number of different types of biomarkers, but one of the most effective methods for identifying highly specific and acutely sensitive biomarkers is through the use of gene- and protein-expression-profiling technologies<sup>151–153</sup>. The advantage of gene-expression studies is that they are large-scale (able to monitor gene-expression changes across an entire genome in one assay), high throughput and highly cost effective (relative to other methods). For example, one of the areas in which this technology has received the greatest attention is in identifying biomarkers for cancer, a field in which expression profiling has been accepted as a powerful tool for identifying specific biomarkers for disease progression, and discriminating between different subtypes of cancer, and, in some cases, identifying biomarkers for susceptibility to specific therapeutics<sup>154–156</sup>.

With regard to infectious diseases, expression profiling of human neutrophils exposed to bacteria reveals dramatic changes in the level of hundreds of mRNA species, including those for cytokines, receptors, membrane-trafficking regulators and genes involved in apoptosis<sup>155</sup>. More importantly, expression profiling of the neutrophil response indicates that key differences in mRNA-expression patterns could be detected on the basis of whether the cells were exposed to pathogenic or non-pathogenic bacteria. Other studies of virus–host interactions using expression technologies and genomic systems studies of host–pathogen interactions have identified specific host factors that pathogens can subvert to optimize their replication and life cycle<sup>154,156</sup>.

Recently, gene-expression-profiling technologies have been applied to the identification of biomarkers for predicting the toxicity of compounds. The field of toxicogenomics has received much interest in both the commercial and academic sectors because of its capability to successfully predict the toxicity of compounds in drug development research, as well as in environmental studies<sup>157</sup>. Existing expertise could be harnessed and applied to developing predictive models to assess the extent of exposure to a biological agent, disease progression and to predict clinical outcomes.

In the future, the creation of a widely available human-gene-expression database of responses to biological threat agents would be extremely beneficial for the rapid and decisive identification of each agent — via a quick and simple blood test. Traditional methods for the identification of biological agents have focused on identifying the agent itself rather than identifying host response. However, many biological agents, such as haemorrhagic fever viruses, could be infectious at levels well below the limit of detection afforded by current technologies. Because the human innate immune system is an exquisitely refined, highly sensitive and highly specific detection system for pathogens, monitoring changes in host innate response via biomarkers is a novel method for identifying exposure to biowarfare agents at very early time points.

### Challenges and future trends

The work reviewed in this manuscript provides evidence that the scientific community has not turned a blind eye to countering biothreat agents, but has responded with a massive effort that has resulted in a steep and productive learning curve. This effort has been facilitated by timely and significant increases in support from funding agencies. However, there is a serious lack of organization in how biodefence is currently addressed. Our existing preparedness and response measures are not sufficient to meet the challenges of a bioterrorist attack<sup>158</sup>. This is due not only to a lack of cooperation and coordination, but also to ineffective detection networks, a lack of time-effective diagnostic methodologies and the dearth of a clear vision and strategy to translate all of the publicly funded biodefence research into useful therapies and antidotes. These issues can be easily mitigated with a unified plan of action, orchestrated by a central entity overseeing a comprehensive and organized approach to biodefence. We foresee such a central entity playing a pivotal role in ensuring that cross-communication between agencies is

facilitated, and that research is focused and completed in a timely manner. In addition, as potential new therapeutics emerge from the drug discovery pipeline, greater involvement from the pharmaceutical industry will be required. It is an accepted fact that the industry is adept at translational research — that is, rapidly and effectively converting potential therapies into approved drugs. However, incentives will need to be put in place to encourage the pharmaceutical industry to conduct such costly studies, and this is where a unifying biodefence entity can have a major facilitating role. Presently, project Bioshield is a start, but needs serious improvements. The ability to develop new therapeutics, and their approval as drugs that can be strategically stockpiled, is urgent. However, new technologies for detecting the release of biothreat agents, and timely protocols for the specific diagnosis of a biothreat agent that has been used, will be needed; this in turn could prevent the chaos that was experienced during the anthrax attacks of 2001. If we start making plans today, and unify our efforts, it will be possible to create a true biodefence shield that will effectively curtail future acts of bioterror.

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Competing interest statement  
The authors declare no competing financial interests.

#### Online links

#### FURTHER INFORMATION

Bioterrorism Resources:  
<http://www.libraryofcongress.gov/bioterrorism.asp>  
CDC Emergency Preparedness & Response Site:  
<http://www.bt.cdc.gov/>  
Centers for Disease Control: <http://www.cdc.gov/>  
Developmental Therapeutics Program NCI/NIH:  
<http://dtp.nci.nih.gov/>  
US Army Medical Research Institute of Infectious Diseases:  
<http://www.usamrid.army.mil/>  
US Department of State: <http://usinfo.state.gov/>  
Access to this interactive links box is free online.



## EXHIBIT E

The government is pouring money into sensors to detect bioweapons, but skeptics question whether they can really protect the public from the array of potential threats

## Up in the Air

Pentagon employees couldn't see the gas seeping into their building. They couldn't taste or smell it. But strategically placed sensors immediately picked up the problem, precisely tracking the wafting gas. Everyone was safe.

This was not reality. This was Pentagon Shield, a Department of Defense exercise last spring that simulated a biological or chemical attack. Research teams released sulfur hexafluoride—a harmless gas used in airflow testing—outside the Pentagon intermittently over several days. Standard gas analyzers traced its movement around and into the building, while other sensors recorded weather conditions. With those data, scientists are refining a computer model of aerosolized weapon movement.

In a real attack, however, unlike a neatly defined exercise, it's unclear how well actual sensors would perform. The Department of Homeland Security (DHS) spends more than \$60 million annually on environmental detectors that monitor outdoor air for bioweapons, but many scientists argue that those detectors are ineffective. Now, DHS plans to spend at least \$32 million more, over the next 18 months, to develop next-generation sensor technology.

"This research has tremendous promise," says Penrose Albright, assistant secretary for science and technology at DHS. But scientists remain skeptical that government contractors really can design sensors that quickly, cheaply, and accurately detect one of the dozens of bacteria, viruses, or toxins that could become aerosolized bioweapons (see table).

### Hazardous history

Bioagents instill fear because just a little can pack a big punch. "Infectious biological agents are on the order of 1000 to 1 million

times more hazardous than chemical [agents]," says Edward Stuebing, head of aerosol sciences at the U.S. Army Edgewood Chemical Biological Center in Edgewood, Maryland.

For decades, these worries were the quiet domain of U.S. military and national weapons labs, funded by the Department of Energy or the Defense Advanced Research Projects Agency. Researchers at Los Alamos National Laboratory (LANL) in New Mexico and Lawrence Livermore National Laboratory (LLNL) in California collaborated on an early biodetection network, dubbed BASIS. That eventually led to the sole environmental bioweapon sensor deployed nationwide today: BioWatch, an aerosol system that works like a vacuum cleaner, sucking air over filter paper that traps aerosol particles. Although earlier BASIS sensors were designed only to detect bioweapons during specific events, such as the Olympics, DHS has deployed BioWatch sensors to continually monitor air in more than 30 major cities.

Despite DHS claims of a perfect record, scientists privy to classified assays suggest that the sensors may experience false positives—mistaking normal environmental toxins for bioweapons. Others complain that because the assay results are classified, they have not been evaluated by outside scientists.

DHS's Albright characterizes BioWatch as a starting point, a relatively cheap system that can be upgraded with new technology. Much of the cost of BioWatch—roughly \$60 million annually, or \$2 million per city—is labor, he says: "Today, we collect the BioWatch filter, take it to the lab, treat the sample, do an initial screen, and then, if we get a hit, take it through an extensive battery of tests."

DHS wants a faster, sleeker system—one that continuously sniffs for bioweapons and

can be sampled frequently with little maintenance, Albright says: "We want high sensitivity, minimal false alarms, and low cost, so we could deploy it nationally in large quantities and expect it to be maintained by, say, volunteer firefighters."

That's a big jump from today's BioWatch. But DHS's external funding arm, the Homeland Security Advanced Research Projects Agency (HSARPA), thinks it can make the leap. The agency recently launched its first research push, allocating more than \$32 million to 14 outside teams.\*

DHS is funding six teams to develop high-priority "detect-to-treat" systems. These would be deployed outdoors like BioWatch but would identify a bioweapon within just 3 hours, enabling doctors to treat exposed civilians. The remaining eight teams are doing feasibility studies for "detect-to-protect" systems, for use inside critical buildings and in specific outdoor spots, to detect a bioweapon within 2 minutes, in time to warn civilians and trigger responses in, say, ventilation systems.

"We are asking everybody to work as fast as they can," says Jane Alexander, deputy director of HSARPA. "In some cases, we have told bidders, 'We know we're asking for the sun, the moon, and four planets. If you can only give us two planets, go ahead.'" With DHS investment, several sensor prototypes probably could be deployed within months, says J. Patrick Fitch, head of chemical and biological national security at LLNL.

### Fine-tuning

To build next-generation sensors, DHS hopes to tweak existing prototypes with the latest technology. Some sensors will run

\* [www.dhs.gov](http://www.dhs.gov)

simultaneous assays on microchips, for instance, or tap new genomic markers for more definitive pathogen signatures.

All biosensors share two basic tasks: to sample air particles and to identify any pathogens. For sampling air particles 1 to 10 micrometers in size, a sensor includes one (or more) of several technologies. A vacuum, for instance, sucks air over filter paper to trap particles, as in the BioWatch sensor. Alternatively, a wetted cyclone draws air down a tube injected with water, which moves with centrifugal force to capture particles. A third variety, called a virtual impactor, uses tiny jets to push air particles down a tube at high speed, concentrating them while diverting excess air. Each differs in cost, sensitivity, speed, and complexity.

For the second task—isolating and identifying bacterial, viral, or toxic particles trapped in the sample—sensor systems typically run immunoassays, polymerase chain reactions (PCR), or mass spectrometry screens. Again, there are tradeoffs. Detect-to-protect technologies are relatively fast and cheap but often carry higher rates of false positives. “If I go from wanting an answer in an hour to wanting one in 2 minutes, I have eliminated all kinds of technologies, like PCR,” says Fitch.

Although slower, the detect-to-treat sensors often use PCR to glean greater detail about a pathogen’s identity, activity, and susceptibility to various treatment options. Among the DHS-funded teams, at least two detect-to-treat prototypes are already being field-tested. One is TIGER—for Triangulation Identification for Genetic Evaluation of Risk—developed by Science Applications International Corp. in San Diego, California, and Ibis, a division of Isis Pharmaceuticals in Carlsbad, California. TIGER works by sampling the air, extracting nucleic acids, and amplifying those acids with broad-based PCR primers that capture all biological agents in the sample. TIGER electrosprays the PCR products into a mass spectrometer that produces each agent’s mass and DNA base composition. Scientists compare an organism’s DNA signature with those in a broad database, confirming its identity—or, in the case of an unknown organism, using phylogenetics to characterize it. This process takes up to a day.

A similar sensor, the Autonomous Pathogen Detection System (APDS), has already been field-tested in the Washington, D.C., Metro transit system and at the San Francisco and Albuquerque airports. LLNL developed this sensor and licensed the technology to MicroFluidic Systems, which leads one of the DHS-funded research teams.

This sensor works by screening air particles with immunoassays or PCR analysis. By multiplexing—or running multiple tests simultaneously—an APDS unit can screen for more than 100 different bacteria or viruses in about an hour. Networked sensors communicate data to a remote console, often via wireless connection, so scientists get monitoring updates from afar. APDS can identify a known bioweapon in 30 minutes to 1.5 hours, Fitch says.

Faster detect-to-protect sensor prototypes are also emerging. One DHS-funded

team could instantly warn that bioagents may be present—and possibly trigger changes in ventilation systems or sound alarms. But the system offers less detail on pathogens than slower varieties do.

#### Wrong track

Still, skeptics question whether DHS’s push for environmental detection is misguided.

Microbiologist Paul Jackson of LANL argues that biosensor research is a costly diversion that will provide, at best, a false sense of security. “Everybody has aerosols on the brain,” he says. “Frankly, I don’t know that environmental monitoring of aerosols at random—or even in important places—is necessarily the best approach.”

Jackson and others argue that more biodefense funds and government guidance should go to hospitals nationwide for “syndromic surveillance” or for the use of simple, reliable blood tests and other diagnostics to detect bioweapons. “The best sentries we have

are patients who come into [emergency rooms] with suspicious symptoms,” Jackson says. If an initial wave of bioterror victims was diagnosed quickly, he adds, many might be saved—and a nationwide alert could immediately be launched.

The federal government has already promised more than \$2 billion in biodefense funds to local public health leaders, and the Centers for Disease Control and Prevention has urged those leaders to invest in syndromic surveillance. But local efforts are patchy—and, many say, poorly coordinated.

DHS also encourages syndromic surveillance. But its detection efforts begin in the environment, where questions first emerge. Did an attack actually happen? Can it be stopped? How can patients be treated? Can buildings be decontaminated?

Tradeoffs are likely to continue. Future bioterror weapons, scientists say, could include genetically engineered pathogens, prions, and bioregulators. All demand new sensors—and questions.

—KATHRYN BROWN

### CDC Category Listing of Select Agents

#### CATEGORY A

- Anthrax
- Botulism
- Plague
- Smallpox
- Tularemia
- Viral hemorrhagic fevers

#### CATEGORY B

- Brucellosis
- Epsilon toxin of *Clostridium perfringens*
- Food safety threats (*Salmonella*, *E. coli*)
- Clanders
- Melioidosis
- Psittacosis
- Q fever
- Ricin toxin
- Staphylococcal enterotoxin B
- Typhus fever
- Viral encephalitis
- Water safety threats (*Vibrio cholerae*, *Cryptosporidium parvum*)

#### CATEGORY C

- Emerging infectious diseases such as Nipah virus and hantavirus



**Close encounters.** Researchers have begun field-testing biosensors in urban subway systems and airports, among other indoor venues.

team leader, Johns Hopkins University’s Applied Physics Laboratory (APL) in Laurel, Maryland, is developing a time-of-flight mass spectrometer that can, within minutes, identify a biological agent based on its proteins or peptides. APL’s sensor automatically sucks in aerosol samples, mixes them with an ultraviolet light-absorbing chemical, and pulses the samples with UV light in a mass spectrometer. Based on light scattering and molecular weight, the system identifies key proteins, say, found in biotoxins. Such a sys-

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